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(54) Title: A METHOD FOR IDENTIFYING A NOVEL MULTIDRUG-RESISTANCE TYPE

(57) Abstract

The subject invention deals with the problem of disease recurrence after the treatment of cancers with drugs that often result in resistance to treatment with such a drug or even with agents to which the patient had not previously been exposed. This phenomenon is known as multidrug-resistance. The main cause for chemotherapy failure is the presence of drug-resistance cancer cells which exist prior to or arise during treatment. We illustrate a method for identifying a novel multidrug-resistance type and a method for inhibiting such drug resistance. Diagnostic kits for use in such methods as well as a method for clinical prognosis are described. The major aspect of the invention is that this novel type of multidrug-resistance is related to the presence of and the amount of a component of a cytoplasmic ribonucleoprotein structure called a vault.

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A METHOD FOR IDENTIFYING A NOVEL MULTIDRUG-RESISTANCE TYPE.

BACKGROUND OF THE INVENTION

It is well-known that many types of cancer can initially be treated with currently available drugs. However, often resistance to treatment with such a drug can occur. In particular if the disease recurs as it does with variable frequency often further treatment with the agent originally used for treatment or even with agents to which the patient has not previously been exposed will occur. This phenomenon is known as multidrug-resistance. The last 20 years a lot of research has been done with regard to understanding the cause of this phenomenon as well as developing methods for circumventing the negative consequences thereof. The main cause for chemotherapy failure is the presence of drug-resistant cancer cells which exist prior to or arise during treatment. In particular the problem is large in lung cancer where survival rates have not improved significantly during the last 4 decades. Small cell lung cancer exhibits a high initial response rate but most relapse and survival rates above 2 years are less than 10% Most importantly the chemosensitizers verapamil and cyclosporin A which show promise for P-gp type drug resistant cells do not elicit response from the lung cancers (WO 94/10303). Drug-resistance observed in the clinic is broad and encompasses various unrelated drugs suggesting concurrent operation of different mechanisms. The understanding of these mechanisms is fundamental to developing strategies aimed at preventing or circumventing resistance thus improving the results of chemotherapy. Studies on the mechanisms of resistance to date have concentrated on cancer cells selected in the laboratory for high levels of resistance by exposure to anticancer drugs.

In addition, drug resistance systems related to topoisomerase II and GSH have been described. Most research has been directed at the MRP and P-gp type MDR and some has been carried out for cisplatin resistance mediated by the GS-X pump. A requirement for alternative methods for the prognosis for various forms of cancer that have been treated with chemotherapeutic drugs that show development of multidrug resistance remains high notwithstanding the large amount of research carried out in this area. In view of the fact that resistance occurring after treatment with cisplatin also occurs quite frequently leading to low recovery rates for patients undergoing chemotherapy research into these two problems is of vital importance for improving cancer therapies. Once the mechanisms for drug resistance

have been elucidated it will then become possible to produce drugs capable of circumventing or perhaps inactivating them to a sufficient degree for chemotherapy to be succesful. Another large problem regarding chemotherapy is the fact that certain tumor types are resistant to treatment with drugs from the outset, i.e. contain natural mechanisms for providing drug resistance.

The mechanisms which enable tumor cells to survive and proliferate in the presence of relatively high concentrations of toxic substances are not fully understood. A great number of in vitro biochemical studies have described differences between parent ("sensitive") and resistant cells selected by exposure to cytotoxic drugs of different chemical classes, i.e. altered enzyme activity, for example 7-dihydrofolate reductase, glutathione-S-transferase, topoisomerase II and stimulated DNA repair. (For a review see Hase J.D. et al. Biochem. J. 1990; 272:281-295.) These differences are thought to account for resistance to distinct or closely related cytostatic drugs. Frequently however upon exposure to chemotherapeutic drugs mammalian cells can acquire resistance to many structurally and functionally unrelated compounds, i.e. display the phenomenon of multidrug-resistance (MDR). The drugs included in the MDR spectrum have different targets and do not share a common metabolic activation or inactivation pathway. Major active anti-cancer agents such as anthracyclines, Vinca alkaloids, podophyllotoxins and actinomycin D belong to this category of drugs. For this reason MDR has been postulated as being due to a decrease of drug concentration at the site of action.

P-gp-MDR.

One mechanism by which mammalian cells can acquire MDR namely the overexpression of a plasma membrane protein, P-glycoprotein (P-GP) has been defined in considerable detail. Treatment of experimental cell lines with a single cytotoxic agent such as an anthracycline or Vinca alkaloid often results in the development of a broad cross resistance to a variety of agents which are distinct, structurally and functionally (Biedler J.H. & Riehm H (1970), Cellular resistance to actinomycin D in Chinese hamster cells in vitro: Crosssresistance, radioautographic and cytogenetic studies. Cancer Res. 30: 1174-1184; Ling V & Thompson L.H. (1974) Reduced permeability in CHO cells as a mechanism f resistance to colchicine. J. Cell. Physiol. 83: 103-116). Research in this area has provided insight into the basis of a similar type of multidrug-resistance (MDR) which occurs in cancer

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patients undergoing chemotherapy. The molecular basis of MDR in many experimental isolates appears to be related to the ability of the cell to greatly decrease drug accumulation (DanÆ K (1973) Active outward transport of daunomycin in resistant Ehrlich Ascite tumor cells. Biochim. Biophys Acta 324; 466-483; Peterson et al (1974) Some biochemical properties of Chinese hamster cells sensitive and resistant to actinomycin D. J. Cell Biol. 63: 223-779; Skovsgaard T (1978) Mechanism of cross-resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. Biochem Pharmacol. 27: 1221-1227). This occurs as a result of overexpression of the MDR1 gene which encodes a transmembran protein P-gp (Endicott J.A. and Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. Ann. Rev. Biochem. 58: 137-171). P-gp can apparently bind various cytotoxic agents and in an energy requiring reaction pump this material from the cell. P-glycoprotein is a member of a superfamily of membrane proteins that serve to transport a variety of molecules ranging from ions to proteins across cell membranes. This superfamily is known as the ATP-binding cassette (ABC) superfamily of membrane transport proteins. (For a review see C.F. Higgins, Ann. Rev. Cellbiol. 8, 67 (1992)). The discovery of P-glycoprotein and its occurrence in a variety of tumor types has stimulated the search for chemosensitizers or reversing agents capable of blocking the function of P-gp and consequently of reversing resistance. Chemosensitizers that can reverse P-glycoprotein mediated multidrug resistance (P-gp-MDR) include verapamil and cyclosporin A.

However since overexpression of P-glycoprotein is lacking in some of the more prevalent forms of cancer, such as lung cancer the high frequency of multidrug resistance exhibited by such cells cannot thus be explained.

Non-P-gp-MDR.

In the past few years evidence has been obtained that cell lines treated with the cytotoxic agent doxorubicin can develop MDR as a result of reduced accumulation of drug however these cells are devoid of detectable levels of P-gp. With regard to non-P-gp MDR a large number of theories exist. Types of non-P-gp MDR have been described which may involve topoisomerases or glutathione transferase. A list of cell lines selected for multidrug-resistance which are defective in drug accumulation in the absence f P-gp is given in Table 1. This list has been derived from Center M.S. Cytotechnology 12: 109-125 (1993) Division

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of Biology, Kansas State University, Manhattan, Kansas 66506, USA; Nielsen D and Skovsgaard T (1992) Biochim Biophys Acta 1139; 169-183.

Cells exhibiting a drug accumulation defect and subsequently shown to be P-gp negative were first described in 1985-1986 with the selection of adriamycin resistant HL60 cells. HL60/AMR, HL60/ADR and an adriamycin resistant human lung cell isolate COR-L23/R. The absence of MDR1 expression in HL60/AR has been documented in studies using the polymerase chain reaction (Gervasoni et al. (1992). Homogeneous staining region in anthracycline-resistant HL60/AR cells not associated with mdrl application. Cancer Res. 52: 1-6). In some cell lines non-P-gp MDR can even be followed by the appearance of P-gp during continued exposure of cells to increasing levels of drug. In the past few years several additional non-P-gp MDR isolates have been obtained such as GLC4/R (Zijlstra et al. (1987). Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res. 47: 1780-1784). SK-MEL-170/R (Panneerselvlam et al 1987) and HT1080/R (Slovak et al. (1987)) derived from human small cell lung common human melanoma and human fibrosarcoma cells respectively. It was found that exposure of these cells to adriamycin results in multiple phenotypical changes but the genetic relatedness of these alterations remains to be determined. The human squamous lung cell line SW1573 exhibits about a 10-fold increase in resistance to adriamycin (SW1573/120) and is negative for MDR1 expression (Versantvoort et al. (1992) Energy-dependent processes involved in reduced drug accumulation in multidrug-resistent uman lung cancer cell lines without P-glycoprotein expression. Cancer Res. 52: 17-23). As there is only modest difference in the rate of efflux of [14C] Daunorubicin from sensitive and resistant cells and although SW1573/120 seems to accumulate less drug than sensitive cells in an energy requiring reaction this particular property may not be related to an enhanced efflux system. Resistance of GLC4R and SW1573/R to agents such as anthracyclines and etoposides is also related to alterations in topoisomerase II (Zijlstra et al. (1987), Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res. 47: 1780-1784; Eijdems et al. (1992), Genetic transfer of non-P-glycoprotein-mediated multidrug resistance (MDR) in somatic cell fusion: dissection of a compound MDR phenotype. Proc. Natl. Acad. Sci USA 89: 3498-3502). Genetic transfer studies with the non-P-gp MDR SW1573/50 using somatic cell fusions demonstrate that distinctive genetic lesions contribute to reduce drug accumulation and altered topoisomerase II (Eijdems et al. (1992), Genetic transfer of

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non-P-glycoprotein mediated multidrug resistance (MDR) in somatic cell fusion: dissection of a compound MDR phenotype. proc. Natl. Acad. Sci USA 89: 3498-3502). A non-P-gp MDR isolate was selected after treatment of the human small lung cell line H69 with adriamycin (Mirski et al. (1987), Multidrug resistance in a human small cell lung cancer cell line selected in Adriamycin. Cancer Res. 47: 2594-2598). According to Cole et al. [(1991), Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. Cancer Res. 51: 3345-3352] these cells do not appear to be defective in cellular drug accumulation as transport studies with radioactively labelled daunomycin, VP-16 or Vinblastine suggest. The cells are however cross-resistant to etoposides and Vinca alkaloids and contain reduced levels of topoisomerase II. Cole et al. went on to further study this phenomenon and cloned from mRNA of H69/AR cells cDNA which encodes a protein having sequence homology with P-gp and other members of a family of proteins which function in a variety of transport processes (Cole et al. (1992), Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258: 1650-1654). This H69 AR exhibits a drug resistance pattern typical of that associated with increased amounts of P-glycoprotein, however it does not overexpress this protein or its encoding mRNA as with most clinical specimens of small cell lung cancer cell lines. Another feature that distinguishes H69 AR from cell lines overexpressing P-glycoprotein is the inability of cyclosporin A and several other chemosensitizing agents to reverse doxorubicin resistance of these cells. A randomly primed cDNA library was constructed from H69AR mRNA and screened by differential hybridization with total cDNA prepared from H69 the parent strain and H69 AR mRNA. Analysis for differential signals was carried out leading to the cDNA encoding MRP designated by Cole et al. as multidrug resistance-associated protein. This was also discovered to be present in a doxorubicin selected Hela cell line and was overexpressed therein. MRP mRNA was not detectable in normal cells of placenta, brain, kidney, salivary glands, uterus, liver and spleen. It was readily detectable in lung, testis and PBMC's. MRP thereby displays a different tissue pattern of expression then P-gp which is highly expressed in secretory organs and tissues such as the adrenal glands, kidneys, lumenal epithelium of the colon and the murine gravid uterus. Cole et al. located the gene coding for MRP on chromosome 16. They could not however exclude the possibility that the amplicon contains additional genes that are maybe responsible for or contribute to the

multidrug resistance phenotype. Although total cellular drug accumulation appears unchanged in H69AR cells the possibility remains according to Cole et al. that sequestration of drugs in these resistant cells may be modified so that the drugs are less able to reach their intracellular sites of action. They consider that the isolation and characterization of MRP provided definitive molecular evidence for the existance of a transporter that may confer or at least contribute to a multidrug resistance in small cell lung cancer. The overexpression of MRP in multidrug-resistant HeLa cells and inherently resistant NSC LC cell lines suggest that this protein could play a role in resistance in other malignancies as well.

Since the original research done by Cole et al. further tests have been carried out. In a Journal of the National Cancer Institute 86 (2) p. 110-117 (1994) Barrand et al undertook a study to clarify the relationship of the 190K protein to MRP gene expression in non-P-glycoprotein containing MDR cells of the large cell and adenocarcinoma lung cancer lines COR-L23 and MOR. They determined that the amount of detectable 190K protein was closely associated with degree of drug resistance. Cell lines surviving at higher drug concentrations have greater amounts of protein and revertant lines grown without drug show reduced expression of the protein together with enhanced drug sensitivity. The amount of MRP messenger RNA correlates closely with that of the 190K protein. They concluded that the 190K protein detected immunologically is likely to be the protein encoded by the MRP gene which becomes overexpressed in these cells as a consequence of chromosomal amplification and fragmentation. However, even though this is associated with drug resistance enhanced drug efflux and decreased drug accumulation in cell lines the role of this protein in clinical resistance has yet to be determined (J. Natl. Cancer Inst. 86: 110-117, 1994).

An antiserum raised against the peptide sequence GTQLSGGQKQRIAIA a peptide which appears in different regions of the P-glycoprotein molecule was used to immunologically detect MRP. This antiserum could be used for detecting the 190K protein in the human leukemic cell line HL60/ADR and also revealed a protein similar in size in the large cell lung cancer line COR-L23/R, however, Barrand et al. were not able to clone the gene coding for this protein based on the synthetic peptide sequence to generate probes. Barrand et al describe how membranes from cells of the drug resistant large cell lung tumor line COR-L23/R were fractionated and the 190K protein enriched membrane fractions were pooled and used to raise antibodies in rabbits. Th resultant antiserum was treated by

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exposure to similar membrane fractions prepared from the sensitive parental cell line COR-L23/P to extract antibodies detecting proteins common to both sensitive and resistant cells, i.e. proteins irrelevant to resistance thereby leaving theoretically only antibodies to proteins differentially expressed in resistant cells. This was used to probe Western blots of membranes from COR-L23/R and MOR/0,2R cells and the predominant protein detectable in membranes of the resistant cells was about 190K in size. This protein was absent from membranes of the sensitive parental cells. Subsequently MRP RNA expression using PCR amplification was detected.

The finding of a strong association between the amount of detectable 190K protein and the level of expression of the MRP gene in the lung tumor cell lines described by Barrand et al however did not link the 190K protein directly to the MRP gene. At least one other protein had also been detected immunologically in P-glycoprotein negative MDR tumor cells that overexpressed the MRP gene. This particular protein had a molecular mass of 110K which argued against it being the product of the MRP gene with the single open reading frame of 1531 amino acids. The 190K protein described by Barrand et al. in MDR lung tumor cell lines is much more compatible with a protein encoded by the MRP gene. It was considered highly likely that the 190K protein detected as a drug resistance associated protein both in lung tumor cells and in leukemic cells (Barrand et al. Br. J. Cancer 65 (SUPPLXVI):21, 1992 and Marquardt D et al. Cancer Res. 50:1426-1430, 1990) is the newly discovered transporter MRP which becomes overexpressed in cells following exposure to doxorubicine as a consequence of chromosomal amplification and fragmentation. And although there is no obvious deficit in drug accumulation in H69 AR cells from which the MRP gene was sequenced the H69 AR cell line does show a similar profile of drug cross resistance including doxorubicin, vincristine, colchicine and etoposide even though the COR-L23/R and MOR/R cells show clear alterations in drug accumulation and increased drug efflux (Twentyman P.R. et al. (1986) Br. J. Cancer 53: 529-537). However it still has to be established whether these changes in drug transport functions are important clinically as doubts have been raised about the status of the MRP as a drug resistance gene (Cole et al. Science 258: 1650-1654, 1982 and Comment in Science 62:879, 1993). There is the possibility that the MRP gene overexpressed in MDR cell lines is simply co-amplified while the real gene responsible for MDR resides with the MRP gene on the same amplicon.

In Cancer Res. 54, 357-361 (1994) Grant C.E. et al. (including Cole and Deeley) carried out transfection of HeLa cells with an expression vector containing the MRP cDNA and illustrated that this resulted in acquisition of resistance to doxorubicin, vincristine and VP-16 but not cisplatin. In PNAS 1994 Zaman G.J.R. et al. show that transfecti n of MRP cDNA into human lung carcinoma cells also results in MDR. They examined the effect of overexpression of MRP on drug resistance and drug accumulation in SW-1573 lung carcinoma cells. They also raised antibodies against segments of MRP and used these to determine the main cellular location of the protein. The question of how MRP acts still remains unresolved. It could simply act like P-gp as a plasma membrane pump extruding drugs seeing as drug accumulation has been reported for several non-P-gp MDR cell lines that were later found to overexpress MRP, however the MDR H69 AR cell line in which the MRP gene was discovered did not illustrate drug accumulation. The drug accumulation for this cell line was reported to be the same as for the parental cell line and Cole et al therefore considered other mechanisms than decreased drug accumulation for MRP action (Cole et al. Science (1992) 258, 1650-1654; Cole et al. (1991) Cancer Res. 51, 3345-3352 and Cole et al. (1992) Can. J. Physiol. Pharmacol. 70, 313-329). The cellular location of MRP does not seem to be similar to that of a plasma membrane transport such as P-gp. The 190KDa protein detected in non-P-gp MDR cells thought to be MRP was mainly found in the endoplasmic reticulum rather than in the plasma membrane (Krishnamachary, N. and Center, M.S. (1993) Cancer Res. 53, 3658-3661 and Barrand M.A. et al. (1994) J. Natl. Cancer Inst. 86, 110-117). The results of Zaman et al. showed that MRP is remarkably similar to the drug transporting P-gp in its mode of action. It can cause resistance to a range of hydrophobic drugs. It is predominantly located in the plasma membrane and can decrease drug accumulation in the cell and increase the efflux of drugs from cells. Therefore, Zaman et al. postulated MRP acts as a drug pump like P-gp extruding hydrophobic compounds from cells against a concentration gradient.

The drug resistance spectra associated with MRP and MDR1 overexpression are described as remarkably similar given the large difference in sequence between MRP and MDR1 (=P-gp). There are however also important differences as MRP provides no resistance to taxol, cyclosporin A does not reverse a decrease of accumulation of drug like it does for P-gp and isoflavanoid G nisteine slightly reduces the decreased drug accumulation in an MRP transfectant. However as this substance is too toxic for patients the search is

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going on for less toxic analogues to overcome MRP MDR. Strangely the modest overexpression of MRP in the transfectant made by Zaman et al. and in the doxorubicin selected SW-1573 variant 2R120 (Kuiper C.M. et al. (1990) J. Cell. Pharmacol. 1, 35-41) results in a higher level of vincristine resistance than the massive overexpression of MRP in the GLC4/ADR line. This cell line is 34 fold more resistant to doxorubicine than its parental cell line but only 3 fold more resistant to vincristine. Possibly post translational modifications determine the activity of MRP to a greater extent than for P-gp. If this is the case some of the non P-gp MDR cell lines displaying only marginal or no increase in MRP mRNA could still contain an increased activity of MRP. It will be necessary to shut off MRP expression by antisense techniques or ribozymes to determine whether non-P-gp MDR phenotype of these lines is due to MRP as well.

There is however little information on the contribution of increased levels of MRP to drug resistance of human cancers and even though high levels of MRP mRNA were found in leukemic cells of a high percentage of patients with chronic lymphocytic leukemia there was no relation to prior chemotherapy or treatment outcome (Burger, H. et al (1994) Leukemia 8: 990-997).

A summary of the results found by Cole et al with regard to MRP can be found in international patent application WO 94/10303 filed on 27 October 1993 with priority date 27 October 1992 and 8 March 1993.

Cisplatin resistance

For sake of clarity it is pointed out that cisplatin is a chemotherapeutic drug not to date considered to be associated with multidrug resistance in the classical sense.

In Toshihisa Ishikawa's article in the Journal of Biological Chemistry, vol. 269, no. 46, 18 November, p. 29085-29090 of 1994 accumulating evidence appears to suggest that cellular GSH (glutathione) is a critical determinant in the tumor cell resistance to chemotherapeutic agents such as nitrogen mustards, chloroethyl nitrosoureas and cisplatin (Meister, M 1991 Pharmacol. Ther. 51, 155-194; Hamilton, T.C. et al. 1965 Biochem. Pharmacol. 34, 2583-2586; Somfai-Relle, S. et al. 1984, Cancer Treat. Rep. 11, 443-454; Arnick B.A. et al. 1984, Cancer Res. 44, 4224-4232) Evidence that several GSH drug conjugates are potentially cytotoxic suggests that the limination of GSH-drug conjugates from tumor cells is an important factor for the cellular toxicity of anticancer drugs (Ishikawa

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T. et al. (1993), J. Biol Chem 268. 20116-20125). They have shown that cisplatin reacts with intracellular GSH and that the resulting glutathione-platinum (GS.Pt) complex is actively exported from leukemia cells via the GS-X pump (Ishikawa T. et al. (1993), J. Biol Chem 268. 20116-20125). Since the GS.Pt complex is a potential inhibitor for protein synthesis, the function of the GS-X pump is considered to modulate the resistance of human cancers to cisplatin (Ishikawa T. et al. (1993), J. Biol Chem 268. 20116-20125; Hamaguchi K. et al. 1993 Cancer Res. 53, 5225-5232).

Cisplatin is an effective antitumor agent for treating various human cancers of the testicle, ovary, brain, head, neck and bladder (Rosenberg et al. 1993 Cancer 55, 2303-2316). Its antitumor activity is attributed primarily to its ability to form DNA-platinum cross-link adducts. Despite its clinical effectiveness, cellular drug resistance is a significant obstacle to a long term, sustained patient response to cisplatin-based therapy.

Ishikawa et al used a cisplatin-resistant variant of human leukemia HL-60 cells as a model system and provide evidence that the GS-X pump is functionally overexpressed in cisplatin-resistant HL-60 cells and that the GS-X pump, (the ATP dependent glutathione S-conjugate export pump) plays a significant part in vesicle mediated excretion of GSH drug conjugate resistant cells. The rate of ATP dependent transport of the GSPT complex measured with plasma membrane vesicles was about 4-fold greater in HL-60/R-CP cells and in HL-60 cells. A number of membrane proteins were overexpressed in these cells whereas P-glycoprotein was not immunologically detected in the membrane preparations from resistant and sensitive cells. They suggested that the expression of the GS-X pump in both cisplatin-resistant and cisplatin-sensitive cells is related to cell proliferation. The GS-X pump is a novel ATP-dependent transporter that mediates the release of glutathione disulfide (GSSG), glutathione S-conjugates, cysteinyl leukotrienes and certain organic anions from normal and cancer cells (Ishikawa et al. 1991 in Structure and Function of Glutathion S-Transferase, pp 211-221 CRC Press Boca Raton). The export of glutathione S-conjugates from cells is important not only in interorgan metabolism of glutathione but also in reducing the intracellular accumulation of potentially cytotoxic GSH conjugates. Thus, the GS-X pump is called the "phase III" detoxification system for biotransformation of endo- and xenobiotics (Ishikawa et al. 1992 Trends Biochem. Sci. 17, 463-468).

In Int. J. Cancer 1993 May 8, vol. 54 (2) p. 309-14 Supino et al described a human small cell lung cancer cell line mutant of N592, a mutant caused by selection on basis

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of resistance to doxorubicin exhibiting an atypical MDR. This atypical MDR included resistance to cisplatin. However, no difference of doxorubicin uptake or efflux could be ascertained despite increased resistance. Verapamil was shown to partially revert resistance without affecting cellular kinetics. These findings were considered to be consistant with undetectable levels of MDR gene expression. Molecular analysis of other putative mechanisms of MDR indicated no difference in GSH levels or GSH related enzymes and a marginal reduction of topoisomerase IIa expression associated with a high topoisomerase I which cannot explain high resistance. This supported the view that alternative unidentified mechanisms existed causing cisplatin resistance and classical multidrug resistance without however offering any teaching of such a mechanism.

Summary of the background art.

Multidrug resistance of the classical type has been associated with MRP and P-gp. In addition, drug resistance systems related to topoisomerase II and GSH have been described. Most research has been directed at the MRP and PG-gp type MDR. Of the classical MDR and other drug resistance systems postulated only P-gp type MDR has been found to have some possible value in a clinical setting (Chan, H.S.L. et al. Journal of Clinical Oncology, Vol. 8, No. 4 (April), 1990, pp 689-704; Dalton, S. et al. Journal of the National Cancer Institute [J. Natl. Cancer Inst. 81:696-701, 1989]). In particular for the P-gp and MRP systems, the classical MDR systems it is plausible that these types of drug resistance only occur in vitro where cells are exposed to exceedingly high levels of drug which will not occur in a clinical setting i.e. during chemotherapy of a patient. To date it therefore remains a puzzle as to whether the occurrence of multidrug resistance, for example through detection of overexpression of MRP or P-gp, can be considered indicative as prognostic for resistance of tumor cells in vivo in a clinical setting.

Apart from the systems described above a large number of proteins has been described as being differentially expressed in particular cell lines that exhibit certain types of resistance to drugs. These are summarized in table 1 and it is reiterated that a large number of these have to date not even been correlated to drug resistance and even more have not been illustrated as being of prognostic value in a clinical setting of MDR of any form let alone of a broader resistance type or cisplatin resistance.

SUMMARY OF THE INVENTION

Quite unexpectedly a number of the above-mentioned problems can now be addressed. The subject invention is related to a novel type of multidrug-resistance that has been discovered. This novel type of drug-resistance is not due to P-gp, MRP, GSH or topoisomerase II. This new type of drug-resistance has been found to comprise a broader range of drugs than the classical MDR drugs described in the state of the art which are commonly linked to the known systems such as described above. In particular it has been found that this new type of drug-resistance can comprise cisplatin resistance. However, besides this resistance to cisplatin resistance to drugs commonly associated with classical MDR like P-gp and MRP MDR also occurs. Another important aspect of this invention is that this novel system appears to occur in a clinical setting. Thereby at last providing markers that can be used for prognosis of treatment with chemotherapy. More especially this resistance appears in relation to an even broader range of drugs than any of the other systems postulated which systems in fact could to date not even be considered as clinical markers for the presence or development of multidrug-resistance. On the basis hereof therefore methods of prognosis, novel and simple methods for determining multidrug-resistance occuring in a cell, test systems for analysis of development of resistance and also development of chemosensitizers able to overcome such resistance have now become possible.

DETAILED DESCRIPTION OF THE INVENTION

What has unexpectedly been discovered is the fact that multi subunit cytoplasmic structures present in a broad range of cell types known as vaults are strongly associated with a novel type of multidrug-resistance. This novel type of resistance has been designated vault related multidrug-resistance (VR-MDR).

Vaults were discovered by Kedersha, N.L and Rome, L.H. J. Cell Biol. 103, 699-709 (1986) when electron microscopy showed small ovoid bodies in rat liver vesicle preparations (Rome L.H., Kedersha, N.L. and Chugani, D.C. Trends Cell Biol. 1, 47-50 (1991)). The structures are ribonucleoprotein particles containing 4 proteins of Mr 210, 192, 104 and 54 KD respectively and a species of small RNA of approximately 140 bases in the

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relative molar ratios of 3:1:55:7:9. The particles measure approximately 35 x 63 nm, have a total mass of 13 MD and are shaped as hollow barrel-like structures with 822 symmetry being composed of two identical cup-like halves joined at their open ends. Vaults have been found in cells ranging from amoeba to man (Rome, L.H., Kedersha, N.L. and Chugani, D.C. Trends Cell Biol. 1, 47-50 (1991) and Kedersha, N.L. et al. J. Cell. Bio. 110, 895-901 (1990)). Approximately 95% of the vaults are localized in the cytoplasm, whereas as little as 5% are associated with the nucleus. Within the nuclear membrane, vaults are thought to function as transporters or central plugs of nuclear pore complexes (NPCs) (Kedersha, N.L. et al. J. Cell Biol. 112, 225-235 (1991) and Kickhoefer, V.A., et al. J. Biol. Chem. 268, 7868-7873 (1993). The precise role of vaults in the cytoplasm to date was unknown, however in Trends. in Cell Biol. vol. 1 August/September 1991 Kedersha et al postulated that there could be a possible functional relationship between vaults and nuclear pore complexes that involves their physical interaction. This interaction could be a docking between vaults and NPCs, with vaults themselves possibly constituting the nuclear pore plugs that are seen associated with a subpopulation of NPC's in isolated nuclear membranes. This raised the possibility that vaults mediate transport between the nucleus and the cytoplasm, where the vast majority of vault particles are located. A definitive role for vaults in nucleocytoplasmic transport, however, awaits the results of ongoing functional studies. Reichelt et al. (J. Cell. Biol. 110, 883-894 (1990) used STEM to determine that the mass of the central plug of the NPC is 13000 kDa, a value identical to the mass of a vault. Analysis of the plug indicates that, like a vault, it has a diameter of 30-35 nm and is composed of two equivalent halves, each with eightfold symmetry (Akey, C.W. (1990) Biophys. J. 58, 341-355). Studies of vaults by freeze-etch microscopy followed by platinum shadowing (Kedersha, N.L. et al. (1991) J. Cell. Biol. 112, 225-235 have revealed a great deal of underlying structural detail. On polylysine-coated mica vaults opened into flower-like structures that were usually seen in pairs, suggesting that an intact vault is composed of two folded flowers. Each flower contains eight rectangular petals surrounding a central ring, and each petal is connected to the ring by a short thin hook. Vaults are highly conserved among vertebrate species and have been purified from the liver of chicken, cow, bullfrog (*Rana catesbeiana*) and the South African clawed frog *Xenopus laevis*, in additi n to the rat (Kedersha, N.L. et al. (1990) J. Cell. Biol. 110, 895-901). Vaults from diverse species are nearly identical in size, shape and morphology. Minor variations have been noted in the size

of the major vault protein, but antibodies raised against rat vaults recognize the major vault protein in all eukaryotic species tested, including man, dog and *Drosophila*. In additi n, vaults have been successfully purified from the vegetative amoeba of the lower eukaryote *Dictyostelium discoideum*. Since the petal represents the largest part of the flowers it seems likely that they are composed of the 104 kDa polypeptide, which constitutes more than 70% of the protein mass. Stoichiometric analysis indicates that there are 96 copies of the 104 kDa polypeptide per vault, and it seems likely that each petal comprises three dimers of the 104 kDa species. Since digestion of the vRNA within vaults with RNase caused no change in either the mobility of the vaults on agarose gels or the structural characteristics assessed by TEM and STEM, it seems unlikely that the RNA plays a structural role. It is likely that the other proteins comprise the remaining structural components, the central rings and hooks.

We have isolated the complementary DNA coding for the major component protein of a vault in humans. A monoclonal antibody was used to screen a cDNA-library derived from the human multidrug-resistance (MDR) fibrosarcoma cell line HT1080DR4 (Slovak, M.L. et al. Cancer Res. 48, 2793-2797 (1988)). A protein with Mr over 110 KD was specifically isolated. The sequence displays a single open reading frame of 2688 base pairs coding for an 896 amino acid protein with a calculated Mr of 100 KD. A computer assisted search of the Swiss protein bank showed strong homology of the isolated protein with the *Dictyostelium discoideum* major vault protein (mVP-a), the protein accounting for more than 70% of the composition of *Dictyostelium discoideum* vaults (accession number p34118). Alignment of the two protein sequences consisting of 896 amino acids for the human mVP and the *Dictyostelium* (mVP) protein with 843 amino acids respectively showed that 57,3% of the amino acids (484) were identical. Taking into account the large evolutionary gap between the lower eukaryote *D. discoideum* and man, this degree of homology makes it highly probable that the isolated human protein and the *D. discoideum* mVP are genetically related. Further searching revealed homology of a little less than 88% with another amino acid sequence namely that of the rat major vault protein (the cDNA nucleic acid sequence is available under GenBank accession number U09870). Kickhoefer, V.A. and Rome, L.H. in Gene 51 (1994) p257-260 describe that rat liver vaults were purified to homogeneity and shown to consist of four protein species (210, 192, 104 an ' 56 kDa) and a unique small RNA. Although the size of the major vault protein varies somewhat between species it retains immunological cross reactivity. The Dd vault unlike mammals

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exhibits two forms of major vault protein with MW 94 and 92 kD repectively. They are known as mVPA and mVPB. The Dd mVPA sequence has been determined.

Kieckhoefer and Rome used a polyclonal antibody directed against the rat vault to screen a rat cDNA library. They describe that searches of GenBank and EMBL databases with FASTA revealed no other sequence matches than with Dd mVPA and that of the 843 aa that overlap 479 are identical, i.e. 57% with a high degree of highly conserved amino acids in the C terminal portion of the protein (aa 649-703) which is consistent with the cross reactivity.

Analysis of the amino acid sequences of the human vault protein and the rat protein revealed a correspondence between 785 amino acids, i.e. homology of 87,7% and a similarity of 23 i.e. 2.6%.

The view that the protein isolated is part of a human vault unit is supported by the observation that precipitation experiments with the MAb directed against the protein (depending on the stringency) allows co-precipitation of proteins with an Mr f approximately 50 kD and approximately 200 kD.

The National Cancer Institute (NCI) has developed a large scale in vitro anti cancer drug screening based on the use of a disease oriented panel of drug unselected human cancer cell lines. Since most of the drug selected MDR cell lines to date show extremely high levels of resistance unlikely to be encountered clinically this NCI panel was considered to provide an excellent in vitro model to test in a more clinically oriented way the relevance of MDR mechanisms in intrinsic drug resistance. Previously the NCI panel was characterized at the functional level by in vitro group sensitivity to a series of MDR related and MDR non-related drugs and P-gp expression was studied. The idea of the current study was to examine the contribution of MRP and the major vault protein to the complex picture of multidrug resistance and further characterize this notable group of cancer cell lines for resistance markers.

The subject invention is directed at a method for identifying a multidrug resistant cell exhibiting multidrug resistance of a novel type, said novel type being other than P-gp-MDR, MRP-MDR, GSH-associated MDR and topoisomerase associated-MDR, said novel type to be called vault related multidrug resistance (VR-MDR), said method comprising determining the presence of and optionally the amount of a component of a cytoplasmic ribonucleoprotein structure called a vault, said component being a protein or an

RNA sequence comprised in the vault in a manner known per se to a person skilled in the art of determining the presence and optionally the amount of a protein or an RNA sequence in a cell. In particular the method according to the invention comprises determining whether a cell exhibits VR-MDR type multidrug resistance to at least one classical non-MDR drug, i.e. to at least one drug not associated with classical MDR, i.e. to a drug not belonging to the category of drugs associated with classical P-gp-MDR or classical MRP-MDR or topoisomerase associated MDR. The term "classical MRP-MDR" as used in this description is to be regarded as the type of multidrug resistance that is non-GSH related MRP-MDR. In particular, the method according to the invention can be used to determine whether said cell exhibits VR-MDR type multidrug resistance, in particular to a drug that does not belong to the category of hydrophobic drugs, such as anthracyclines, vinca alkaloids, epipodophyllotoxins and any other type of drug commonly associated with multidrug resistance of the classical type, in particular with P-gp-MDR or MRP-MDR. Such a drug can belong to the group consisting of thioguanine, 6-mercaptopurine, 5-fluorouracil, 5-FUDR, hydroxyurea, 1-ß-D-arabinofuranosylcytosine, bisulfan, nitrogen mustard, chlorambucil, melphalan, CCNU, cisplatin, CBDCA, carboplatin, BCNU, bleomycin and any other drug with hydrophilic characteristics equivalent to those of the just mentioned compounds, which will be apparent to a person skilled in the art. In particular, resistance to platina-comprising drugs can be determined in the method according to the invention.

An example of a manner known per se for determining the presence and optionally the amount of the protein or RNA sequence in the cell is a method for determining the presence and optionally the amount of a nucleic acid sequence in the cell in a manner known per se to a person skilled in the art. Such a manner known per se can, for example, comprise nucleic acid amplification techniques which are commonly known in the art such as polymerase chain reaction, NASBA, reverse chain amplification or the RNAse protection assay of said nucleic acid sequence or a part thereof, said nucleic acid sequence either encoding the protein that is a component of a vault or said nucleic acid sequence being the RNA sequence comprised in the vault. In particular, the nucleic acid sequence to be determined is preferably the mRNA encoding a component of a cytoplasmic ribonucleoprotein structure called a vault. The component most pref rably to be determined is the major vault protein. The means for carrying out the amplification are common to a person skilled in the art and can be found in most handbooks f molecular biology. There ar

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also many kits available on the market for carrying out such reactions. What is required for such amplification techniques, however, are amplification primers and detection probes.

The nucleic acid sequence of the cDNA encoding the major vault protein of a human vault has been determined by us and is represented in sequence listing no. 1. A person skilled in the art armed with this information can subsequently determine any nucleic acid sequence with which the amplification reaction can be carried out. In general terms a nucleic acid sequence will be required capable of hybridizing to the nucleic acid sequence of sequence listing no. 1 or the complementary sequence thereof under conditions commonly used for amplification reactions or nucleic acid detection. Usually normal to stringent hybridisation conditions are applied. Preferably such a primer or probe will exhibit a degree of homology as close as possible to the sequence to which it must hybridize, i.e. a part of the sequence of sequence id no. 1 or the complementary sequence thereof. But less homology is possible. Preferably the nucleic acid sequence will comprise codons preferably found in humans. Preferably the homology will be higher than 60%, preferably 70%, with more preference for higher than 80%, even higher than 90% and most preferably within a range of 95-100%. In particular the nucleic acid sequence will preferably encode an amino acid sequence exhibiting more than 87,7% homology with sequence id no. 2 and most preferably being identical to the corresponding part of the nucleic acid sequence no 1. The length of a nucleic acid sequence primer to be used in an amplification reaction will also be obvious to a person skilled in the art, generally speaking such a primer will have a minimum length of 12 to 21 nucleotides. Variations in length are possible and it will be obvious to a person skilled in the art which lengths are operable in the amplification reaction selected. The person skilled in the art will also be able to assess the minimum length required for a detection probe.

The nucleic acid sequence to be determined in a method according to the invention encodes a protein that is the component of a cytoplasmic ribonucleoprotein structure called a vault, said vault having the biological activity of conferring multidrug resistance on a drug sensitive cell when a protein is present in the cell and in particular when the protein is overexpressed in the cell, said multidrug resistance being of the VR-MDR type. The major component of such a vault structure is a suitable component. Said protein will preferably comprise the amino acid sequence of sequence corresponding to EMBL Genbank accession number X79882 nucleic acid sequence or an equivalent amino acid

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sequence. An equivalent amino acid sequence comprises one or more silent mutations whilst maintaining the functionality of the protein comprising sequence id. no 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault. Mutations of one amino acid are known generally not to specifically alter the activity of a protein. Conservative amendments of certain amino acids exhibiting similar charge and hydrophobicity do not seriously alter protein characteristics and such amendments and mutations are considered to be included in the amino acid sequences and the nucleic acid sequences according to the invention which encode such amino acid sequences. The nucleic acid sequence to be determined can also encode a protein comprising a part of sequence id. no. 2 or a part f the equivalent amino acid sequence which comprises one or more silent mutations, said part encoding a protein maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault. In particular an amino acid sequence or a nucleic acid sequence encoding an amino acid sequence falls within the scope of the invention when exhibiting homology higher than 87,7 % with the amino acid sequence of sequence id no 2 or the amino acid sequence encoded by sequence id no 1. A nucleic acid sequence encoding the rat major vault protein in which typical human codons have replaced the rat codons of the nucleic acid sequence of GenBank accession no. U09870 also falls within the scope of the invention.

As indicated the major vault protein of *Dictyostelium discoideum* exhibits less than 60% homology with the sequence derived for the human major vault protein and therefore it is expected that organisms more closely related to humans will exhibit a higher degree of homology. This is corroborated by the sequence given for the rat major vault protein exhibiting homology of 87,7% at amino acid level. The method is therefore also directed at determining multidrug resistance of a cell as stated above, wherein the manner known per se for determining the presence and optionally the amount of the protein or the RNA sequence in the cell comprises determining the presence and optionally the amount of a nucleic acid sequence in the cell wherein said nucleic acid sequence encoding the protein exhibits at least 60% homology with the nucleic acid sequence of sequence id. no. 1, preferably homology larger than 70%, more preferably larger than 80%, with more preference for homology larger than 90% and most preferably 95-100%. As the method is generally most useful for determining MDR f human cells the preference exists for the nucleic acid sequence to exhibit maximum homology, preferably somewhere in the regi n

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between 80-100%. The nucleic acid sequence should preferably encode a protein exhibiting more than 87,7% homology with the amino acid sequence of sequence id no. 2.

From a comparison between the *Dictyostelium discoideum* major vault protein sequence and/or the rat protein sequence and the human vault protein areas of conserved regions can be estimated and preferably nucleic acid sequences comprising such regions r being capable of hybridizing thereto or to the complementary sequence thereof will be preferably used as primers in an amplification reaction or as probe in a detection reaction, wherein said detection reaction need not necessarily occur of amplified nucleic acid, but may also be used for detecting relevant non amplified nucleic acid in other types of assay commonly known in the art of molecular biology. The nucleic acid sequence encoding the protein that is to be determined in a manner known per se in a method according to the invention can be an equivalent nucleic acid sequence encoding the amino acid sequence of sequence id. no. 2, said equivalent being derivable by a person skilled in the art from the degeneracy of the genetic code. The nucleic acid sequence to be determined can also comprise a part of sequence id. no. 1 or the equivalents thereof said equivalents being due to the degeneracy of the genetic code, said part encoding a protein maintaining the functionality of the protein comprising amino acid sequence id no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault. A part maintaining such functionality will generally have a length similar to the natural protein and is preferably longer than 75% of the amino acid sequence of sequence id no 2. The types of mutations generally expected to maintain the protein activity of the natural protein have been described above.

The protein to be determined will be encoded by a nucleic acid sequence of any of the types described above, said nucleic acid sequence being capable of hybridising under normal to stringent hybridisation conditions to a nucleotide sequence encoding an amino acid sequence according to sequence id. no. 2 or being capable of hybridizing under normal to stringent hybridisation conditions to the complementary strand of the nucleotide sequence encoding an amino acid sequence according to the sequence id. no. 2, said nucleic acid sequence having a length sufficient to provide a protein maintaining the functionality of the protein comprising sequence id. n . 1, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault. In addition the protein to be determined can be encoded by a nucleic acid sequence according to any of the above categories, said nucleic acid

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sequence being capable of hybridizing under normal to high stringency conditions t a nucleic acid sequence according to sequence id. no. 1 or being capable of hybridizing to a complementary strand of the nucleic acid sequence id. no. 1 or being capable of hybridizing to a part of the nucleic acid sequence according to sequence id. no. 1 or to a part of the complementary strand of the nucleic acid sequence according to sequence id. no. 1, said part having a length sufficient to provide protein maintaining the functionality of the protein comprising amino acid sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault. In short, any nucleic acid sequences that encode a protein that can be considered an equivalent of the protein with the amino acid sequence according to sequence id. no. 2 can be determined in a method according to the invention. Determination of the range of sequences that fall within the scope stated will be apparent to a person skilled in the art without undue experimentation.

A method for identifying a multidrug resistance cell exhibiting multidrug resistance of a novel type, said novel type being other than P-gp-MDR, MRP-MDR, GSH-associated MDR and topoisomerase associated MDR, said novel type to be called VR-MDR comprising determining the presence of and optionally the amount of a component of a cytoplasmic ribonucleoprotein structure called a vault, said component being a protein or an RNA sequence comprised in the vault in a manner known per se for determining the presence and optionally the amount of a protein or an RNA sequence in a cell can comprise a determination in a manner known per se of the degree of binding that occurs between a detectable molecule and the cell or part of the contents of the cell, said molecule being capable of specifically binding to the nucleic acid sequence encoding the protein that is a component of a cytoplasmic ribonucleoprotein structure called a vault. Another option is the molecule being capable of specifically binding to the complementary sequence of the nucleic acid sequence encoding the protein that is a component of a cytoplasmic ribonucleoprotein structure called a vault, examples of such nucleic acid sequences have been given above, i.e. encoding the amino acid sequence according to sec. id. no. 2 or the nucleic acid sequence according to sequence id. no. 1. The molecule will exhibit the highest homology with said nucleic acid sequence and will be at least 60% homologous as stated previously in the description for the amplification primers. Hybridisation under normal to stringent conditi ns will occur. A detectable molecule that can be used in such a method will for example be a nucleic acid capable of specifically recognizing the nucleic acid sequence to be determined

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and can for example be any of the nucleic acid sequences described above or a part thereof comprising at least 10 consecutive nucleotides of such a nucleic acid sequence. The length required of such nucleic acid sequence will be apparent to a person skilled in the art. In particular, a detectable molecule will be capable of hybridizing under normal to stringent conditions to any of the nucleic acid sequences encoding a protein that is a component of a cytoplasmic nucleoprotein structure called a vault that have been described above, i.e. the sequence capable of hybridizing to the nucleic acid sequence, according to sequence id. no. 1 and any of the equivalent sequences in the various variations described.

Another alternative for a method according to the invention arises when said molecule is capable of specifically binding to the nucleic acid sequence which is the RNA sequence comprised in the vault or is the complementary strand of the RNA sequence comprised in the vault. In short a detectable molecule can be a nucleic acid strand capable of hybridizing under normal to stringent hybridisation conditions to a nucleic acid sequence encoding the protein that is a component of a cytoplasmic rubonucleoprotein structure called a vault or the complementary strand of such a nucleic acid sequence or to the RNA sequence comprised in a vault or the complementary strand of such an RNA sequence.

The detectable molecule for detecting any nucleic acid sequence encoding the protein that is a component of a cytoplasmic ribonucleoprotein structure called a vault or the RNA sequence comprised in the vault as described can be an antibody and more preferably a monoclonal antibody that is capable of specifically recognizing and binding to such a nucleic acid sequence.

A method for identifying a multidrug resistance cell exhibiting multidrug resistance of the VR-MDR type, said method comprising determining the presence of and optionally the amount of a component of a cytoplasmic ribonucleoprotein structure called a vault, said component being a protein or an RNA sequence comprised in the vault in a manner known per se for determining the presence and optionally the amount of a protein or an RNA sequence in the cell can be carried out by determining the degree of binding that occurs between a detectable molecule and the cell or part of the contents of the cell, said molecule being capable of specifically binding to the protein or part of the protein that is the component of a cytoplasmic ribonucleoprotein structure called a vault. Preferably, such a component comprises an amino acid sequence of sequence id. no. 2 or an equivalent sequence thereof as described above. Th detectable molecule can for example be an

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antibody and will most preferably be a monoclonal antibody. Polyclonal antibodies capable of recognizing vault proteins are known as is apparent from the already cited article of Kedersha et al. Another antibody that can be suitably used is a monoclonal antibody designated LRP 56 which has already also been previously disclosed in the art as recognizing the lung resistance protein, a protein known to have molecular weight 110 kD and to be expressed in multidrug resistant cells of a non-P-gp type. No link has previously been made between the monoclonal antibody LRP 56 and major vault proteins. An antibody specific for an epitope of a protein having biological activity conferring VR-MDR type multidrug resistance on a drug sensitive cell when the protein is present in the cell and forms a vault, can be provided with a detectable marker in a manner known per se such as a fluorescent group, an enzyme, radioisotope or can be capable of being immobilized in a manner known per se and the subsequently labelled and/or immobilized product can be detected also in a manner known per se to a skilled person in the field of immunoassays in a method according to the invention. The same procedure can be used when the antibody is to detect a nucleic acid sequence as described previously.

Another embodiment of the inventive concept comprises a method for inhibiting VR-MDR type drug resistance of a cell comprising inhibiting in a manner known per se production and/or activity of a protein in said cell, said protein being a component f a cytoplasmic ribonucleoprotein structure called a vault. The protein being a component of a cytoplasmic ribonucleoprotein structure can comprise any of the equivalents described above in the method for identifying a multidrug resistance cell of the VR-MDR type. The inhibition in a manner known per se of production of the protein can occur for example by down regulating initiation of expression of the protein. In an embodiment of this aspect of the invention the production of the protein can be inhibited by contacting the cell with a molecule that is the antisense nucleic acid sequence of the nucleic acid sequence encoding the protein or contacting the cell with a molecule that is the antisense nucleic acid sequence of a part of the nucleic acid sequence encoding part of the protein, said part being sufficiently long for the antisense nucleic acid sequence thereto to inhibit production of the protein in a manner known per se. Antisense technology is well-known to a person skilled in the art and the reaction conditions or assay requirements will be obvious and not place an undue burden to a person skilled in the art.

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The use of ribozymes such as hammerhead ribozymes or RNA catalytic ribozymes is also an option besides the use of antisense RNA. The technology of use of ribozymes has been described previously in Br. J. Cancer 919940 70, p 239-243 by Holm P.S. et al. wherein P-gp MDR was reversed by introduction of a hammerhead ribozyme. As ribozymes can be targeted to specific RNA sequences they have proved to be useful tools for inhibiting undesired gene expression. The expressed ribozyme decreased the level of mdr1 mRNA expression, inhibited formation of P-gp and drastically reduced daunorubicin resistance. Development of a ribozyme capable of recognizing the nucleic acid encoding the major vault protein of humans as predicted from the amino acid sequence id. no. 2 and analogous application thereof are now possible.

A method for inhibiting VR-MDR type drug resistance of a cell can comprise contacting the cell in a manner known per se with a molecule which binds to a component of a ribonucleoprotein structure called a vault such as a protein forming part of said vault, said molecule preferably being specific for said component and said molecule in particular being an antibody. Such inhibition will require inhibition of the vault, this can be achieved by inactivating all components of the vault. Preferably a method requiring inactivation of the minimum number of components will be carried out. The major component of the vault appears to be the logical target for inactivation.

As stated above, an antibody capable of recognizing a major vault protein is described in the state of the art. Kedersha et al referred to antibodies capable of recognizing various vaults of eukaryotic source. They have not described monoclonal antibodies, th ir antibodies are polyclonal. The monoclonal antibody LRP 56 has been found by us to be an antibody capable of specifically recognizing the human major vault protein and as stated above the link between major vault protein and LRP 56 had not been made previously. In particular any antibody capable of recognizing the amino acid sequence of sequence id. no. 2 or a part thereof can be used in a method according to the invention in particular for human cells in a manner known per se for detecting protein.

Due to the insight that is the basis of the invention it has now become possible to develop a method for protecting a drug sensitive cell from cytotoxity due to exposure to a drug to which it is sensitive, said drug being a drug for which VR-MDR type and optionally also one or more other MDR type resistances occurs, said method comprising increasing the amount r activity of cytoplasmic ribonucleoprotein structures called vaults. One could think

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perhaps of inserting complete vault structures in a sensitive cell or inserting nucleic acid sequences encoding components of such vaults.

The amount and/or activity of the compon nts of a cytoplasmic ribonucleoprotein structure called a vault can be obtained for example by specifically transfecting the cell in a manner known per se with a nucleic acid sequence being or encoding a component of the cytoplasmic ribonucleic protein structure called a vault or a functional equivalent of said component, said nucleic acid sequence or cell comprising regulatory elements enabling production of the component or the equivalent by the cell. The nucleic acid sequence can comprise a nucleic acid sequence encoding a protein of the types disclosed above in the method for determining multidrug resistance having the functional equivalence of a vault component, required for exhibiting multidrug resistance.

The method for protecting a drug sensitive cell from cytotoxicity can be used in combination with chemotherapy techniques so that drug sensitive cells, i.e. non-tum rous cells are not damaged by increased dosages of toxic substances used in chemotherapy to destroy tumors for example when particular healthy cell types are known to be sensitive to a specific drug but such drug has to be used in chemotherapy to attack a tumor increasing the resistance of healthy sensitive cells to this drug can allow the treatment to be carried out without adverse effect to the patient. A person skilled in the art on the basis of equivalent experimentation having been carried out will realize which types of tumors and which locations are suitable for such treatment. In particular haemopoietic cells or bone marrow. Stem cells are suitable targets in which vaults are to be inserted or to be produced to a higher degree. Such cells can be treated increasing the vaults content thereof in a patient who can subsequently be subjected to high dose chemotherapy without the normal resulting damage to bone marrow or haemopoietic cells. The cells can be treated ex situ and be introduced in the patient prior to chemotherapy. The vaults can be isolated from vault comprising cells as such or be produced through recombinant technology.

Another aspect of the invention is formed by a test system comprising a first cell or organism comprising a nucleic acid sequence encoding a protein that is a component of the cytoplasmic ribonucleoprotein structure called a vault or a functional equivalent of said protein, said nucleic acid sequence of th first cell comprising regulatory elements enabling production of the protein by the first cell and said second cell being of a corresponding type of cell to the first cell differing only in production of less protein r the presence of less and

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preferably the presence of none of the protein such that said first cell exhibits multidrug resistance of the VR-MDR type and the second cell either exhibits multidrug resistance of a non-VR-MDR type, for example the classical P-gp type or MRP type or both or preferably the second cell exhibits no multidrug resistance. Preferably the first cell will overexpress the protein. Most preferably the cell will comprise an over abundance of vaults. The nucleic acid sequence can comprise a sequence as described above encoding a component of a vault, in particular the major vault protein encoded by sequence id. no. 1 or the amino acid sequence id. no. 2 or a functional equivalent thereof. Such a test system can comprise a first cell which is a recombinant cell and a second cell which is the corresponding non-recombinant host cell, i.e. the wild type cell. The recombinant host cell can comprise a nucleic acid sequence encoding a protein that is a component of the cytoplasmic ribonucleoprotein structure called a vault or a functional equivalent of said protein, said nucleic acid sequence also comprising regulatory elements enabling expression of the nucleic acid sequence by the cell and said nucleic acid sequence preferably being a nucleic acid sequence encoding the major vault protein of sequence id. no. 1 and so the recombinant host cell expresses more of the protein then the non-transfected host cell, i.e. than the wild type host cell, said recombinant host cell exhibiting VR-MDR type multidrug resistance and said wild type not exhibiting said VR-MDR type of multidrug resistance.

Another aspect of the invention is formed by a method for identifying a substance to which VR-MDR type resistance is conferred comprising contacting a test system as described above with the substance to be tested and determining whether resistance is conferred for said substance in any of the cell types in a manner known per se for drug testing in equivalent systems. In particular, the determination of whether VR-MDR resistance is conferred for said substance in any of the cell types can occur with the method for determining in a manner according to the invention. Such a method being much simpler and easier to carry out quickly than the currently used methods for determining classical MDR. Such a classical method comprises culturing cells subjecting them to the presence of the drug to be tested and monitoring the growth of such cells during a period of approximately 2 - 4 days. This is a time consuming and complicated method which is not suitable for clinical use.

The invention also comprises a method for identifying a substance capable of overcoming multidrug resistance of VR-MDR type comprising contacting recombinant host

cell as described above or a test system as described above or any cell for which VR-MDR type resistance has been determined and determining whether any of the cells are sensitive to said substance in a manner known per se for drug testing in other systems.

It is now possible to identify cells which are rich in vaults. These cells can subsequently be used in testing for drugs that can overcome VR-MDR by monitoring whether such drugs reduce activity or number of vaults.

Also a method for identifying a substance incapable of producing resistance f the VR-MDR type comprising contacting a recombinant host cell as described above or a test system as described above with the substance to be tested in a manner known per se for drug testing in other MDR systems and determining whether any of the cells remain sensitive to said substance is also part of the invention.

What is also claimed is the nucleic acid sequence encoding a protein being the component of a cytoplasmic ribonucleoprotein structure called a vault, said vault having the biological activity of conferring multidrug resistance on a drug sensitive cell when the protein is present in the cell, in particular when the protein is present in the cell, in particular when the protein is overexpressed in the cell, said multidrug resistance being of the VR-MDR type and said protein preferably comprising the amino acid sequence of sequence id. no. 2. Said protein can also be an equivalent amino acid sequence derived from the amino acid sequence of sequence id. no. 2 comprising one or more silent mutations maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault or a part of sequence id. no. 1 or a part of the equivalent amino acid sequence, said part encoding a protein maintaining the functionality of the protein comprising sequence id. no. 1, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault. The nucleic acid sequence claimed is not a known nucleic acid sequence encoding the known *Dictyostelium discoideum* major vault protein or the known rat major vault protein. A nucleic acid sequence encoding a protein being a component of a cytoplasmic ribonucleoprotein structure called a vault, said vault having the biological activity of conferring multidrug resistance on a drug sensitive cell when the protein is present in the cell in particular when the protein is overexpressed in the cell, said multidrug resistance being of the VR-MDR type and said protein preferably comprising the amino acid of sequence id. no. 2 or an equivalent amino acid sequence derived therefrom (e.g. comprising one or more silent mutations maintaining the

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functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault) or a part of sequence id. no. 1 or a part of the equivalent amino acid sequence thereof, said part encoding a protein maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault, wherein the nucleic acid sequence exhibits at least 60% homology with the nucleic acid sequence shown in sequence id. no.1, preferably homology larger than 70%, more preferably larger than 80%, with more preference for homology larger than 90% and most preferably 100% homology is claimed. A nucleic acid sequence of any of the groups claimed comprising the nucleic acid sequence of sequence id. no. 1 or an equivalent nucleic acid sequence encoding the amino acid sequence of sequence id. no. 2, said equivalent being derivable from degeneracy of the genetic code or encoding a part of sequence id. no. 2 or an equivalent thereof, said part encoding a protein maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault is claimed. A nucleic acid sequence as claimed wherein said nucleic acid sequence is capable of hybridizing under normal to stringent hybridization conditions to a nucleotide sequence encoding an amino acid sequence according to sequence id. no. 2 or being capable of hybridizing under normal to stringent hybridisation conditions to the complementary strand of the nucleotide sequence encoding an amino acid sequence according to sequence id. no. 2, said nucleic acid sequence having a length sufficient to provide a protein maintaining the functionality of the protein comprising the sequence id. no. 1, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault also falls within the scope of the invention. In particular amino acid sequences exhibiting 100% homology in the consensus regions of *Dictyostelium*, rat and human major vault protein and maintaining the biological activity of the human protein as such and in combination with the other parameters given for amino acid sequences according to the invention are included in the scope of the invention, as one the nucleic acid sequences encoding such amino acid sequences. Further, a nucleic acid sequence according to any of the preceding four groups further being capable of hybridizing under normal to high stringency conditions to a nucleic acid sequence according to sequence id. no. 1 or being capable of hybridizing to the complementary strand of the nucleic acid sequence according to sequence id. no. 1 or being capable of hybridizing to a part of the nucleic acid sequence according to sequence id. no. 1 or to a part of the complementary strand of the

nucleic acid sequence according to sequence id. no. 1, said nucleic acid sequence having a length sufficient to provide a protein maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault is included within the invention. As is a nucleic acid sequence antisense to any of the five groups of nucleic acids sequences claimed. A nucleic acid sequence according to the invention can also comprise a nucleic acid sequence antisense to a sequence encoding the major vault protein of *Dictyostelium discoideum* or a rat or antisense to a part of such a protein encoding sequence sufficiently long for inhibiting production of a major vault protein in an antisense inhibition method, preferably for inhibition of production of a human major vault protein.

A recombinant vector comprising any of the nucleic acid sequences claimed according to the preceding paragraphs suitable for transformation of a host cell, said vector preferably being an expression vector, further comprising a regulatory sequence operatively linked to the nucleic acid sequence is comprised within the invention. In particular when such a nucleic acid sequence is the sense sequence for a protein being a component f a ribonucleoprotein structure called a vault, such a recombinant vector is an embodiment of the invention that is suitable to be used to transform a host cell and subsequently use said cell in a method for preparing a protein, said method comprising culturing the transformed host cell described above in a suitable medium until the protein is formed and optionally isolating the protein in a manner known per se to a person skilled in the art of protein production. For example suitable host cells will comprise cells normally sensitive to anti-cancer drugs when delivered in high dosages required to damage (resistant) tumor cells. Suitable host cells will be bone marrow and haemopoietic cells.

In addition, the subject invention is directed at the production of novel antibodies capable of being applied in the methods according to the invention disclosed. Such an antibody can be an antibody specific for an epitope of a nucleic acid sequence encoding a protein having biological activity conferring VR-MDR type multidrug resistance on a drug sensitive cell when the protein is present in the cell and forms a vault. Preferably an antibody according to the invention is a monoclonal antibody. A monoclonal antibody according to the invention as claimed does not include LRP 56. Said protein, for example being the major vault protein of sequence id. no. 2 or a functional equivalent thereof as described elsewhere in this description. The antibody can also be an antibody specific for an

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epitope of a protein, said protein encoded by a nucleic acid sequence encoding a major vault protein with amino acid sequence id. no. 2 or a functional equivalent thereof as disclosed elsewhere. Such an antibody coupled to a substance having toxical therapeutic activity whilst retaining the ability to bind the epitope falls within the scope of the invention. Although known antibodies disclosed in the state of the art now known to recognize a number of major vault proteins, for example, monoclonal antibody LRP 56 or the polyclonal antibodies disclosed by Kedersha et al. Journ. of Cell Biology 110: 895-901 as such are not claimed, when the known antibodies are combined with a substance having toxical therapeutic activity such combination is claimed to fall within the scope of the invention by virtue of the fact they recognize a protein associated with the novel type of resistance called VR-MDR and can be used in therapy or knocking out resistance. Suitable antibodies according to the invention or for use in any embodiment of the invention can easily be prepared by immunizing a test animal with isolated vault protein or other component of a vault and subsequently collecting antibodies produced against such a component. If the production in large amounts of monoclonal antibodies is required the preparation can further comprise producing hybridomas that can make the desired monoclonal antibodies. Such technology is well-known to a person skilled in the art and although it can comprise a lot of work does not place an undue burden on a person skilled in the art as it requires application of techniques currently well-known and standard.

What is also claimed is a composition suitable for administration to a mammal, preferably of a human, said composition comprising an antibody specific for an epitope of a protein having biological activity conferring VR-MDR type multidrug resistance on a drug sensitive cell when the protein is present in the cell and forms a vault, said protein preferably being a major vault protein in particular being encoded by a nucleic acid sequence encoding a vault protein with amino acid sequence id. no. 2 or a functional equivalent thereof as defined previously in the description in a pharmaceutically acceptable carrier, thereby rendering the composition suitable for administration to a mammal, preferably a human is also included within the scope of the invention.

A method for clinical prognosis comprising analysis of the occurrence of VR-MDR in a cell or tissue sample of a patient, preferably in a manner described according t the invention for determining whether a cell is VR-MDR multidrug resistant, for example with regard to prognosis for leukemia and acute myeloid leukemia, in particular for lung

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cancer, breast cancer, sarcoma's, colon cancer, renal cancer, testicular cancer, neuroblastoma, rhabdomyosarcoma, and especially ovarian carcinoma and equating occurrance of VR-MDR or an increase in VR-MDR determined from such analysis with chance of poor response to chemotherapy and adverse outcome. As indicated in the introductory portion the problems in treatment of lung cancer are great and now possibilities arise for improving therapy and/or preventing drug resistance. The means for carrying out such a method will be apparent to a person skilled in the art especially after analysis of the examples provided in the description. In particular, such a method is suitable when the chemotherapy is carried out with or is to be carried out with at least one non-classical MDR drug not belonging to the category of drugs associated with classical P-gp MDR, classical MRP-MDR which as indicated elsewhere in the description means non GSH related MRP-MDR, or with the category of drugs not associated with classical MDR, in particular not belonging to the category of classical MDR associated hydrophobic drugs such as anthracyclines, vinca alkaloids and epipodiphyllotoxins. Especially when chemotherapy is carried out or is to be carried out with at least a non classical MDR drug belonging to the group consisting of thioguanine, 6-mercaptopurine, 5-fluorouracil, 5-FUDR, hydroxyurea, 1-ß-D-arabinofuranosylcytosine, bisulfan, nitrogen mustard, chlorambucil, melphalan, CCNU, cisplatin, CBDCA, carboplatin, BCNU, bleomycin and any drug with hydrophilic characteristics equivalent to those of the afore-mentioned compounds, in particular platina comprising drugs such as cisplatin such a method is considered applicable. As is apparent from the examples such a method can be carried out wherein the analyis is immunohistochemical and the antibody is an antibody specific for an epitope of a protein having biological activity conferring VR-MDR-type multidrug resistance on a drug sensitive cell when the protein is present in the cell and forms a vault in particular when the protein is the major vault protein.

The invention is directed at a diagnostic kit for determining whether a cell exhibits VR-MDR type multidrug resistance, said kit comprising a primer or a number of primers necessary for carrying out nucleic acid amplification, said number being dependent on the type of amplification to be carried out and being apparent to a person skilled in the art of amplification reactions of nucleic acid. In particular, such a diagnostic kit would be suitable for use in a method for determining whether a cell exhibits VR-MDR type multidrug resistance in a manner according to the invention and also in a method for prognosis of

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development of VR-MDR in particular for prognosis regarding the outcome of treatment with chemotherapy and overall survival. Such a kit should be suitable for use for any of the embodiments of the two groups of methods described in this description. In particular, the primer or primers will be suitable for carrying out nucleic acid amplification of a nucleic acid sequence encoding a protein being a component of a cytoplasmic ribonucleoprotein being component of a cytoplasmic ribonucleoprotein structure called a vault, said vault having the biological activity of conferring multidrug resistance on a drug sensitive cell when the protein is present in the cell, in particular when the protein is over abundant in the cell, for example, is overexpressed in the cell, in particular when the multidrug resistance is of the VR-MDR type. Such a protein can comprise the amino acid sequence of sequence id. no. 2 or an equivalent amino acid sequence derived therefrom, comprising one or more silent mutations, maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resistance conferring activity when comprised in a vault or a part f sequence id. no. 2 or a part of the equivalent amino acid sequence thereof, said part encoding a protein maintaining the functionality of the protein comprising sequence id. no. 2, i.e. exhibiting the multidrug resitance conferring activity when comprised in a vault or said primer comprising a nucleic acid sequence encoding a protein as just described or a part thereof, said part comprising at least 10 consecutive nucleotides of said nucleic acid sequence, preferably comprising 15 consecutive nucleotides. Said kit can optionally comprise further components known per se for carrying out an amplification reaction of nucleic acid, said kit optionally comprising a container with a control cell of the type to be tested. Such a control cell should be non-VR-MDR. The kit can also further comprise a detection probe for the amplified sequence of a type known to a person skilled in the art.

An alternative diagnostic kit for carrying out determination of whether a cell exhibits VR-MDR drug resistance or determining the prognosis of treatment through chemotherapy can simply comprise a detection probe of nucleic acid encoding a major vault protein for detection of nucleic acid in a classical manner as known before advent of amplification procedures. Such a detection probe has been defined for the amplification embodiment elsewhere in the description. The kit can optionally comprise a container with a control cell of the type to be tested and/or an instructi n manual.

Another embodiment of a diagnostic kit as described above for determining whether a cell exhibits VR-MDR like multidrug resistance, in particular for use in a method

for determining whether a cell is VR-MDR resistant or for determining the prognosis resulting from chemotherapy treatment in any of the embodiments of the method according to the invention disclosed above, comprises an antibody being an antibody specific for an epitope of a protein having biological activity conferring VR-MDR type drug resistance on a drug sensitive cell when the protein is present in the cell and forms a vault for carrying out an immunohistochemical analysis for the presence and optionally the amount of a component of a vault in a sample. Said kit should further comprise at least one additional component required for carrying out the method for determining whether the cell is VR-MDR resistant or for carrying out the prognosis according to the invention. Such an additional component can for example be an instruction manual. The diagnostic kits according to the invention can for example comprise one or a number of cell types known not to be VR-MDR which can serve as reference cells for carrying out a method according to the invention. The kit can comprise any antibody capable of recognizing a major vault protein, in particular any monoclonal antibody.

In the case of cells already being VR-MDR it is also possible for a test kit to comprise such a cell or a range of such cells in order to test drugs for their capability of reducing the resistance. Such a test kit may also comprise antibody according to the invention or LRP 56. Preferably the antibody will be monoclonal.

Another embodiment comprises an antibody capable of recognizing a nucleic acid sequence being or encoding a component of a vault.

The antibodies in any of the kits can already be provided with detectable markers or these can be supplied separately and combined prior to or during the method in which the kit is to be used.

EXAMPLE 1

In Cancer Research 52, 3029-3034, 1992 Lin Wu et al. describe how disease oriented panels of human tumor cell lines used by the National Cancer Institute for large-scale in vitro anticancer drug screening were evaluated for multidrug-resistance phenotype at the functional (in vitro drug sensitivity) and molecular levels. Several tumor cell lines exhibited a high degree of resistance to MDR drugs and relative sensitivity to non-MDR drugs, and contained high levels of MDR-1 mRNA, encoding P-gp and expressed cell surface P-glycoprotein detectable with one or more monoclonal antibodies. Parallel

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expression of all of these features representing the classic MDR phenotype was observed among members of the colon and renal tumor panels. Certain individual cell lines among other panels (lung, ovarian, melanoma and central nervous system) also manifested some aspects of the MDR phenotype to various extents. A total of 61 human tumor cell lines from 8 cancer types (leukemia, central nervous system, melanoma, breast, ovary, colon, lung and renal formed the basis of this study. Chemosensitivity was assessed using a sulphurodamine B assay as described previously. GI_{50} values were calculated. For each drug relative cell line sensitivities were measured by ranking the log (GI_{50}) values (averaged over at least 3 different experiments) for all the cell lines; the rank of 1 was assigned to the cell line with the lowest average log(GI_{50}). Subsequently, for each cell line, an MDR rank as a measure of its relative resistance to MDR related drugs was calculated by averaging the rankings associated with 7 MDR related drugs (actinomycin D, vinblastine sulfate, vincristine sulfate, daunomycin, adriamycin, VP-16 and AMSA). For each cell line also a non-MDR rank (as a measure of its relative resistance to classically MDR unrelated drugs) was determined by averaging the rankings of 15 MDR unrelated drugs (thioguanine, 6-mercaptopurine, 5-fluorouracil, 5-FUDR, hydroxyurea, 1-ß-D-arabinofuranosylcystosine, busulfan, nitrogen mustard, chlorambucil, melphalan, CCNU, cisplatin, carboplatin, BCNU, and bleomycin). Levels of MDR1 mRNA were estimated relative to ß-actin mRNA using a modification of the RNA PCR method of Fuqua et al., as described. P-gp expression was investigated by immunocytochemistry using the avidin-biotin complex method with a panel of 3 MABs: JSB-1, C219, and MRK-16. Slides were semiquantitatively evaluated. Major vault prot in and MRP specific monoclonal antibodies were employed. Immunocytochemistry analysis was performed on acetone fixed (10 min) cytospins. Slides were preincubated with normal rabbit serum for 15 min. and then incubated with major vault protein Mab and MRP- Mabs respectively for 1 hour. Rabbit anti-mouse biotin conjugate (1:150 for 30 min; Zymed Laboratory Inc., San Francisco, CA) or rabbit anti-rat biotin conjugate (1:100 for 30 min; Dako, Copenhagen, Denmark) and horseradish-streptavidin (1:500 for 1 hr; Zymed Lab.) were the second and third step, respectively. Amino-ethyl-carbazole (ICN Biochemicals, Aurora, Ohia) was used as a chromogen. Slides were counterstained with hematoxylin. Negative control slides were used in which all the steps were repeated substituting the primary antibody by an irrelevant mouse IgG or PBS. SW-1573 and SW-1573/2R120 cell lines were kindly provided by Dr H.J. Broxterman (Free University Hospital, Amsterdam,

The Netherlands) and GLC4S and GLC4/ADR cell lines were kindly provided by Dr E.G.E. de Vries (University of Groningen, The Netherlands). These served as controls for the major vault protein and MRP expression. Evaluation was done blind in coded slides. A semiquantitative "staining index" was calculated as the product of the percentage of positive cells and the average staining intensity qualitatively estimated on a scale from 1(+) to 3(+++). Between three and six (median 4) tests for each cell line were used for calculation f average staining index. The same scoring system was used for P-gp expression.

Relation between two continuous variables was assessed by the Spearman's rank correlation coefficient. Difference between the means of two independent groups of observations was determined by the two sample t test. The Welch's test for equality of means (variances are not assumed to be equal) was used when appropriate for more than two independent groups of observations. The data were analyzed using the BMDP statistical software package (BMDP Statistical Software, Inc. Los Angeles, CA).

The results of drug sensitivity are shown for each cell line of the NCI panel in Table 2 as are the mRNA MDR-1 levels P-gp, MRP and mVP protein expression.

The Spearman's rank correlation analysis assessing the association between MDR rank and non-MDR rank yielded a coefficient of 0.78 ($P < 0,0001$) (Fig. 1).

The staining indexes of the MRP antibodies were strongly correlated (Spearman's rank coefficient 0,84, $P < 0,0001$), supporting both, the MRP specificity of these two MAbs raised against different parts of the MRP molecule and the accuracy of the staining index assessment. Calculations were performed using staining indices from both antibodies.

Figure 2 summarizes the results of the expression of MRP and mVP obtained in the study also data on P-gp expression are shown for comparison. MRP and mVP were detected at varying levels in 88% and 78% of the cell lines, respectively. The mean staining index value was 0.82 (range 0,00 to 2.40) for MRP and 0,80 (range, 0,00 to 2.70) for mVP. These MDR-associated proteins exhibited a positive skewed distribution, with the majority of cell lines showing low or moderate expression, substantially lower than in the MDR cell lines GLC4/ADR and SW-1573/2R 120 used as MRP and mVP control, respectively. This feature was more evid nt in the case of MRP, whereas a larger number of cell lines displayed high mVP expression. The sensitivity of the immunocytochemical assay was sufficient to

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detect the reduced level MRP and mVP present in the parental cell lines GLC4 and SW-1573, respectively.

Figure 3 shows examples of MRP and mVP staining and illustrates different scores of the staining index. For both, heterogenous protein expression within an individual cell line was common. Ultimately, in some cases, only a small percentage of cells showed strong MRP or mVP staining among a majority of negative cells. The pattern of staining of the MRP MAB was predominantly cytoplasmic with occasional membranous immunoreactivity, whereas the other MRP-Mab MRP R6 showed a distinct plasma membrane staining in most MRP expressing cells. The mVP Mab displayed a characteristic coarse granular cytoplasmic staining.

Occasionally large vesicular-like cytoplasmic structures were mVP positive irrespective of the fixation method, for example in SK-OV3 ovarian carcinoma cell line.

Correlations of expression of MDR associated proteins with MDR-rank, non-MDR rank and log(GI_{50}) values of a number of classical MDR-related (doxorubicin, vincristine, VP-16, and mitoxantrone) and classical MDR-unrelated (cisplatin, carboplatin and melphalan) drugs are summarized in Table 3. Data on P-gp are given for comparison. MRP staining index yielded Spearman's rank correlation coefficients of 0,12 (P = 0,36) and 0,11 (P= 0,41) with MDR and non-MDR ranks, respectively. The same figures for mVP were 0,40 (P= 0,002) and 0,36 (P = 0,005). MRP showed significant correlation with resistance to vincristine (Spearman's coefficient 0,37, P = 0,004), whereas mVP was correlated with resistance to vincristine and carboplatin (Spearman's coefficients 0,37 (P = 0,004) and 0,38 (P = 0,002), respectively). In addition, lower coefficients, but significant or marginally significant, were found for mVP correlation with VP-16, cisplatin and melphalan.

As low expression of a resistance mechanism, still functionally relevant, may be under the limit of detection of a 48 hrs. chemosensitivity assay obscuring any potential correlation between these two parameters this issue was addressed by selecting a staining index cut-off to distinguish "low" from "high" expressing cell lines for each MDR-associated protein (fig. 3). MVP expression above 0,60 was significantly associated with higher relative resistance to MDR related drugs (t test; P = 0,004), and to MDR unrelated drugs (t test; P = 0,01) (Fig. 3). In addition, mVP expression above 0,60 was specifically correlated with resistance to vincristine (P = 0,02), carboplatin (P = 0,01), and melphalan (P = 0,03). No

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significant cut-off value was found for MRP expression, neither with MDR rank (Fig. 3), non-MDR rank, or with individual drugs.

The panel of cell lines was divided in three equally large groups according to their MDR rank: low, intermediate, and high, respectively (Fig. 4). The cell lines with low MDR rank had a significant lower expression of mVP (mean staining index ± SD was 0,28 ± 0,48) as compared with the intermediate and high MDR rank groups (1,19 ± 0,84 and 0,91 ± 0,78 respectively; Welch test, F = 10,45, P = 0,0003). A number of the most sensitive cell lines were negative for mVP. In contrast, complete overlap with regard to MRP expression was found among the three groups (low, 0,78 ± 0,62; intermediate, 0,70 ± 0,60; high, 0,98 ± 0,65; Welch test, 1,00, P = 0,38).

Thirty-nine cell lines (64%) of this panel expressed more than one MDR-associated protein: 11 (18%) mVP + MRP + P-gp; 26 (43%) mVP + MRP; 2 (3%) mVP + P-gp. Spearman's rank coefficients between the different resistance markers were: mVP versus P-gp, 0,36 (P = 0,009); mVP versus MRP, 0,31 (P = 0,02); MRP versus P-gp 0,10 (P = 0,10). Analysis of the effect of combined expression of the different MDR-associated proteins (by adding the different staining indices for each cell line) on drug sensitivity was performed by Spearman's rank correlation. No improvement with respect of the results obtained with mVP alone was observed, except for a stronger correlation between co-expression of MRP and mVP with resistance to vincristine (coefficient 0,43 P = 0,0008).

DISCUSSION

MDR selected cell lines survived treatment with escalating doses of drugs through several rounds of stringent selection, thus making it problematic to extrapolate the results obtained to the clinic. Such traditionally MDR selected cell lines show very high levels of cross-resistance to natual product and other polycyclic drugs, the so-called classic MDR related drugs, while retaining collateral sensitivity to other agents, the so-called MDR unrelated drugs, and indeed, the resistance mechanism or mechanisms triggered by the selection procedure used in previous experiments may explain the major part of the MDR phenotype as registered to date. In contrast, most human cancers showed a broader pattern of lower levels of intrinsic clinical resistance to both groups of drugs, suggesting that different mechanisms are operative simultaneously. The high correlation found in this study

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between MDR rank and non-MDR rank indicates that a concomitant degree of relative resistance to MDR related and MDR unrelated drugs, exists across this diverse group of cell lines. This in fact reflects clinical experience with cancers from which these cell lines have been derived better than previously postulated models.

This is the first study describing the expression of two new MDR-associated proteins, namely MRP and mVP in a large panel of unselected cell lines, extending the previous report on P-gp expression. In contrast to P-gp which was detected at, in general, very low levels in 24% of the cell lines, MRP and mVP were constitutively expressed and vary in levels in 87% and 78% of the cases, respectively. Since most cancers from this panel, including melanoma, non-small cell lung cancer, CNS tumors, and colon/renal carcinomas, are intrinsically resistant to chemotherapy, high prevalence appears to be a pre-requisite for a resistance mechanism to be clinically relevant. These results thereby supported a role for mVP in intrinsic resistance of cancer, whereas P-gp may play a more general role in acquired resistance after exposure to chemotherapy, (without excluding its cooperation in intrinsic resistance of untreated P-gp positive cancers).

The potential of individual MDR-associated proteins was investigated for identifying drug resistance in this panel of unselected cell lines. mVP expression, determined either by actual staining index (Spearman's correlation) or level of expression (t test) was found to be significantly correlated with resistance to both MDR related and MDR unrelated drugs. mVP by itself may explain a small percentage (» 15%) of the whole variability in relative resistance to both groups of drugs, a finding that seems remarkable considering the complexity of this model. Furthermore, the significantly reduced expression of mVP in the low MDR rank cell lines indicates its presence above certain limits is consistently linked with intermediate and high levels of MDR. Besides its association with global resistance ranks, mVP expression appeared to be specifically linked with resistance to vincristine, carboplatin, and to a lesser degree, with VP-16, melphalan and cisplatin. No correlation with mitoxantrone resistance was found.

The correlation of mVP with resistance to the classical MDR related drugs is in agreement with its overexpression in MDR cell lines, and as it is now translated into drug unselected cell lines, further strengthens the potential of mVP as a clinical marker of MDR. The value of mVP to identify a non-classical MDR phenotype was totally unexpected on the basis of the spectrum of drug resistance of non-P-gp overexpressing MDR cell lines. This

finding can be explained by the strong correlation between MDR and non-MDR ranks among the NCI screen, and suggests that in unselected cell lines mVP could frequently be co-expressed with classical MDR mechanisms, therefore, converting mVP into an indirect marker of this type of resistance as well. In addition, a more direct implication in resistance to some classical MDR unrelated drugs has to be presumed. In particular, the relatively strong correlation with carboplatin (considered as a classical MDR unrelated drug even though little data concerning carboplatin sensitivity in classical MDR cell lines is available) resistance deserves a great deal of attention.

The prevalence of mVP expression in clinical cancer specimens was comparable to that in the NCI panel, and the ability thereof to identify both an MDR of the classical type and a non-MDR phenotype, has been confirmed in clinical studies in acute myeloid leukemia and advanced ovarian carcinoma, respectively (see following examples). The mVP expression was significantly associated with a poor response to chemotherapy, shorter progression-free survival and, in the ovarian carcinoma study, a shorter overall survival. Interestingly, most ovarian carcinoma patients received carboplatin based chemotherapy. In contrast to mVP, no correlation was found between MRP espression and MDR as might have been expected considering the ability of the MRP gene to confer the classical MDR phenotype as illustrated in the literature of the state of the art.

The observation that the high MRP expression in a number of the most sensitive cell lines occurs raises the possibility that the activity of MRP may not be a simple functi n of its concentration, but that it could also be present in an inactive form. The activity of ABC transporters, i.e. P-gp and MRP, once expressed may be regulated, i.e. phosphorylation regulates activity of the cystic fibrosis gene product CFTR and P-gp. Also, it has been suggested that the functional significance of P-gp processing may be particularly important in non-selected, constitutively expressing cancer cells and tumors with post-translational modifications perhaps playing a role in regulation of activity of MRP as well. In support of this view, a post, translationally modified MRP was found to correlate with drug resistance in SW-1573 cell lines selected for low levels of resistance. This possibility could be what has further complicated attempts to correlate clinical resistance to changes in MRP expression which had to date been suggested as potential methods of markers f r classical MDR. A role for MRP in intrinsic resistance as suggested by high prevalence among the NCI screen, can however not be excluded. The high MRP expression among the cell lines

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with the highest MDR rank supports a role for MRP in these cell lines. Furthermore, MRP was specifically correlated with resistance to vincristine, in agreement with the preferential resistance to vincristine observed in MRP transfected cell lines. Also the distinct MRP staining on the plasma membrane observed in many cell lines is compatible with a role for MRP as drug efflux pump in unselected cancer cell lines as well. In addition, the universal cytoplasmic immunoreactivity indicates the presence of MRP in intracellular structure where it could be involved in drug redistribution inside the cell. However, if the subsequent processing of MRP is significant then more detection of MRP expression or overexpression will not serve as a method for prognosis of MDR of the classic type.

Here, we have demonstrated that in unselected cell lines concurrent expression at relatively low level (certainly lower than in the classic MDR cell lines) of different MDR mechanisms is common. This seems to better reflect the clinical setting, and partially differs from MDR cell lines where mVP or MRP are in general mutually exclusive with P-gp. In fact, in series of MDR cell lines with increasing levels of resistance, a shift from mVP or MRP to P-gp occurs at high levels of selection. Co-expression of mVP or MRP and P-gp has been found using clinical samples from patients with acute myeloid leukemia. So, regulation of resistance could be similar in unselected cell lines and in vivo, but need not necessarily be comparable to drug selected MDR cell lines.

The concomitant expression of different MDR mechanisms suggests that they could simultaneously contribute to MDR, although the relative importance of each one may differ in the various cell lines. This, and the lack of correlation for MRP and P-gp alone may account for the failure to detect an improved correlation (with respect to mVP alone) with resistance in the combined analysis. Only the combination of mVP and MRP showed a stronger correlation with resistance to vincristine than either protein alone. Within such a complex system, attempts of disecting the drug resistance phenotype by correlating individual MDR mechanisms or combination thereof with resistance to specific drugs can help to design optimum chemotherapy protocols according to the expression of such mechanisms in clinical specimens. Cancers expressing mVP can be expected to show particular resistance to vincristine and carboplatin and those expressing MRP could also be considered specially vincristine resistant.

In conclusion therefore the NCI human tumor panel screen as used in the study can be considered a more clinically oriented in vitro model for evaluating new mechanisms

of resistance. Its further characterization for resistance markers can assist in better defining mechanisms of resistance of new drugs and providing a basis for more rational drug design.

In our model, cooperation of different processes appears to be common, with mVP playing a major role in identifying a broad spectrum of drug resistance, in particular a broader spectrum of drug resistance than was to date commonly accepted as being the classical multidrug resistance.

EXAMPLE 2

Epithelial ovarian carcinoma is the fourth cause of cancer death in women and the leading cause of gynecologic cancer mortality (Boring CC, Squires TS, Tong T. Cancer statistics, 1993. CA Cancer J Clin 43:7-26, 1993). Approximately 75% of women present with surgically incurable advanced disease. Their treatment with platinum-based chemotherapy after cytoreductive surgery has improved response rate and survival, but the complete pathological response and long term survival rates are still only 20-40% and 10-20%, respectively (Neijt JP, ten Bokkel Huinink WW, van der Burg MEL, et al. Long-term survival in ovarian cancer. Eur J Cancer 27:1367-1372, 1991; Ozols RF. Treatment of ovarian cancer: current status. Sem Oncol 21:1-9, 1994). Chemotherapy failure may be due to drug-resistant cancer cells which exist prior to or arise during treatment (Johnson SW, Ozols RF, Hamilton TC. Mechanisms of drug resistance in ovarian cancer. Cancer 71:644-649, 1993). Drug resistance observed in clinical ovarian carcinoma is broad and encompasses a variety of unrelated drugs (i.e. platinum compounds, alkylating agents, natural products), suggesting concurrent operation of different mechanisms of resistance (Johnson SW, Ozols RF, Hamilton TC. Mechanisms of drug resistance in ovarian cancer. Cancer 71:644-649, 1993) (See introduction of the description).

To date, most clinical studies in ovarian carcinoma have failed to demonstrat a role for any of these mechanisms as a major determinant of response to chemotherapy and survival (7-10).

The aim of the study was to investigate the expression of MRP and mVP, in addition to Pgp, in advanced ovarian carcinomas, and to determine whether such expression was predictive of response to chemotherapy and survival.

METHODS

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Samples selection

Freshly frozen samples of ovarian specimens from 57 women undergoing initial surgery for advanced ovarian carcinoma (stage III and IV according to the International Federation of Gynecologic Oncologists [FIGO] classification [International Federation of Gynecology and Obstetrics: Changes in definitions of clinical staging for cancer of the cervix and ovary. Am J Obstet Gynecol 156:236-241, 1987]) were obtained from diagnostic histopathology laboratories at the Free University Hospital, Amsterdam, and the University Hospital, Groningen during the period 1984-1993 (52 cases after 1987). The samples for study were selected in sequence only for availability of frozen material. All frozen samples had been stored in liquid nitrogen until use. Diagnosis was based on conventional morphologic examination of paraffin-embedded specimens and tumors were classified according to the WHO classification (Serov SF, Scully LH, Sobin LH. Histological typing of ovarian tumors. Geneva: World Health Organization, 1973, 17-18). Carcinomas were classified into well (grade I), moderately (grade II), and poorly (grade III) differentiated (Sobre B, Frankendal B, Veress B. Importance of histological grade in the prognosis of epithelial ovarian carcinomas. Obstet Gynecol 59:567-573, 1982).

Patients

The first column of table 4 shows the clinicopathological characteristics of the 57 patients included in this study. The median age at diagnosis was 66 years (range, 29-84). The patients included had no other form of cancer. All patients were primarily treated with debulking surgery and post-operative chemotherapy, which consisted of a platinum-based regimen in 50 patients (in five patients plus an anthracycline) and a single alkylating agent in seven (Table 4). Response to induction chemotherapy was assessed by second-look surgery or clinical/radiographic evaluation according to WHO criteria (World Health Organization. Handbook on reporting results of cancer treatment. Geneva:WHO, 1979) after 5-6 and 10-12 cycles of platinum-regimen and alkylating agent, respectively. Eight patients had no clinically evaluable disease after initial surgery and did not undergo second-look, and were, therefore, considered not evaluable for response. Of 49 patients evaluable for respons to chemotherapy, eight (16%) achieved a complete response (seven pathologically verified), 17 (35%) achieved a partial response (13 pathologically verified), and 24 (49%) had no

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response (10 pathologically verified). Thus, 20 out of 25 (80%) of the responses were assessed by direct inspection at second-look laparotomy and pathologically confirmed. Patients with incomplete response to induction chemotherapy and patients with recurrent tumors were treated with a variety of second-line chemotherapy regimens. Follow-up for the 57 patients included in the survival analysis was updated in May 1994 (median follow-up was 18 months; range 1-111). At that moment, 38 patients had died of ovarian carcinoma and 19 were alive (median follow-up for the last group was 31 months, range 7-111).

Monoclonal antibodies (MAbs)

Pgp expression was studied with two MDR1-gene specific murine MAbs: MRK-16 (IgG2) which recognizes an external epitope of Pgp was kindly supplied by Dr. Tsuruo (Hamada H, Tsuruo T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells revealed by monoclonal antibodies. Proc Natl Acad Sci USA 83:7785-7789, 1986), and JSB-1 (IgG1) which reacts against an internal epitope of Pgp was produced in our laboratory (Scheper RJ, Bulte JWM, Brakkee JGP, et al. Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glucoprotein associated with multidrug resistance. Int J Cancer 42:389-384, 1988). Both MAbs have been w ll characterized and extensively utilized in the literature, and are considered to yield better results in cryostat sections than in paraffin-embedded sections (Weinstein RS, Kuszak JR, Kluskens LF, et al. P-glycoproteins in pathology: the multidrug resistance gene family in humans. Hum Pathol 21:34-48, 1990). For immunochemical detection of MRP the rat MAb MRPr1 (IgG2a) was developed in our laboratory (Flens MJ, Izquierdo MA, Scheffer GL, et al. Immunochemical detection of MRP in human multidrug-resistant tumor cells by monoclonal antibodies. Cancer Res 54:4557-4563, 1994). MRPr1 was raised after immunization with a fusion protein containing a segment of 168 amino acids in the amino-proximal half of the MRP protein, and has been well characterized by protein blot analysis, immunocytochemical (including specific detection of MRP in S1 cells transfected with a full length cDNA of the MRP gene), and immunohistochemical studies. MRPr1 does not cross-react with the human Pgps (Flens MJ, Izquierdo MA, Scheffer GL, et al. Immunochemical detection of MRP in human multidrug-resistant tumor cells by monoclonal antibodies. Cancer Res 54:4557-4563, 1994). For the investigation of mVP expression we used the

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murine MAb mVP-56 (IgG2b) that was obtained in our laboratory after immunization with the non-Pgp MDR human lung cancer cell line SW-1573/2R120 (Scheper RJ, Broxterman HJ, Scheffer GL, et al. Overexpression of a 110 kD vesicular protein in non-P-glycoprotein mediated multidrug-resistance. Cancer Res 53:1475-1479, 1993). Both MRPr1 and mVP-56 display better immunoreactivity in cryostat than in paraffin-embedded sections.

Immunohistochemistry

Immunostaining was performed on acetone-fixed (10 min) cryostat sections. Slides were preincubated with normal rabbit serum for 15 min and then incubated with MRK-16 1:200, JSB-1 1:100, MRPr1 1:50 or anti-mVP 1:500, for 1 hr. For Pgp and MRP detection an avidin-biotin complex procedure was used in which rabbit anti-mouse biotin conjugate (1:500 for 30 min; Zymed Laboratories Inc., San Francisco, CA) or rabbit anti-rat biotin conjugate (1:100 for 30 min; Dako Corp., Glastrup, Denmark), and horseradish-streptavidin (1:500 for 1 hr; Zymed Lab.) were the second and third steps, respectively. For mVP detection a single-step indirect immunoperoxidase method was employed with affinity purified rabbit anti-mouse IgG conjugated to horseradish-peroxidase (1:25 for 30 min; Dako Corp.). The selection of the immunoperoxidase method used for each MAb was based on previous optimization of the immunohistochemical protocols. Staining with an irrelevant mouse IgG and an isotype matched rat IgG were routinely performed as negative control procedure for the murine and rat MAbs, respectively. Slides were counterstained with hematoxylin. Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as a chromogen.

Cytospin preparations of tumor cell lines (squamous carcinoma KB-3-1, small cell lung cancer GLC4, and non-small cell lung cancer SW-1573) and their correspondent MDR sublines overexpressing Pgp (KB-8-5) (Akiyama SI, Fojo A, Hanover J, et al. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somatic Cell Mol Genet 11:117-126, 1985), MRP (GLC4/ADR) (28), and mVP (2R120) (Kuiper CM, Broxterman HJ, Baas F, et al. Drug transport variants without P-glycoprotein overexpression from a human squamous lung cancer cell line aft r selecti n with doxorubicin. J Cell Pharmacol 1:35-41, 1990), respectively, served as controls for the immunohistochemical assays. The sensitivity of our assays allowed the detection of the low

levels of Pgp, MRP, and mVP present in KB-8-5, GLC4, and SW-1573 cells, respectively (Scheper RJ, Broxterman HJ, Scheffer GL, et al. Overexpression of a 110 kD vesicular protein in non-P-glycoprotein mediated multidrug-resistance. Cancer Res 53:1475-1479, 1993; Flens MJ, Izquierdo MA, Scheffer GL, et al. Immunochemical detection of MRP in human multidrug-resistant tumor cells by monoclonal antibodies. Cancer Res 54:4557-4563, 1994; Akiyama SI, Fojo A, Hanover J, et al. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somatic Cell Mol Genet 11:117-126, 1985).

The slides were examined and scored independently by two observers without knowledge of the clinical information regarding the patients. By prior agreement, samples were scored as positive if more than 10 % of the tumor cells were stained (for Pgp both with MRK-16 and JSB-1). In selected cases with equivocal results, the staining was repeated to ensure reproducibility. The evaluation of immunoreactivity with MRK-16 and JSB-1 was found to be particularly difficult due to the presence of some background staining with the avidin-biotin complex method. Therefore, in order to further confirm the specificity of the reaction we repeated the study of Pgp expression in 42 selected specimens in which th immunoperoxidase method was not completely satisfactory using a sensitive double alkaline phosphatase monoclonal anti-alkaline phosphatase (APAAP) method (30).

Statistical analysis.

Data analysis was performed using the BMDP statistical software package (BMDP Statistical Software, Inc., Los Angeles, CA). Clinico-pathological parameters determined at diagnosis were assessed for their relationship with Pgp, MRP, and mVP expression. Treatment response rates were compared between Pgp, MRP and mVP expression groups, and between the different groups that resulted of combining the expression of these proteins. Qualitative variables were analyzed using Fisher's exact 2-tailed test or the chi-square test, as appropriate. Progression-free and overall survival were measured in months, from the month of surgery to a documented relapse or death, respectively. Actuarial survival curves were stimated using the Kaplan-Meier method (Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457-481, 1958), and differences in survival between subgroups were compared

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with the log-rank test (Mantel N. Evaluation of survival data and two new rank order statistics arising in its considerations. Cancer Chemother Rep 50:163-170, 1966). Multivariate analysis was performed with a Cox proportional-hazards stepwise regression model (Cox DR, Oakes D. Analysis of survival data. London: Chapman & Hall, 1984); the limits of the p values to enter and remove the variables into or from the model were 0.10 and 0.15, respectively. Hypotheses were evaluated at a significance level of 0.05. Two-sided statistical tests were used in all analyses.

RESULTS

Expression of Pgp, MRP and mVP in ovarian carcinomas

Table 4 summarizes the expression of MDR-related proteins and their correlation with different clinicopathological features. Nine out of the 57 patients (16%) were Pgp positive, 39 (68%) were MRP positive, and 44 (77%) were mVP positive. There was no statistical difference in prognostic factors between tumors depending on the expression of MDR-related proteins (for all comparisons $p > 0.05$) (Table 4).

Pgp immunoreactivity was cytoplasmic and in some cases it was also localized to the plasma membrane. MRP staining was predominantly observed at the cell membrane, but some cytoplasmic staining was also present. The intensity of the reactions varied from weak to moderate (more similar to GLC4 than to GLC4/ADR cells), suggesting that in ovarian carcinoma the cellular level of MRP is relatively low or modest. mVP expression was detected as a characteristic granular cytoplasmic, in general strong, staining.

The expression of all three MDR-related proteins was heterogeneous. However, whereas the number of Pgp positive cancer cells was more variable and in general below 50%, MRP and mVP positive cases usually showed immunoreactivity in the majority of cancer cells. Stromal cells, such as macrophages, were occasionally positive for Pgp and MRP but more frequently showed strong immunoreactivity for mVP. In agreement with this observation, mVP-related vaults have been reported to be abundant in macrophages (Rome L, Kedersha N, Chugani D. Unlocking vaults: organelles in search of a function. Trends Cell Biol 1:47-50, 1991).

Expression of Pgp, MRP and mVP and response to chemotherapy.

Table 5 summarizes the correlation between the expression of the three MDR-related proteins and response to induction chemotherapy in 49 patients in whom response was evaluable. There was no significant correlation between Pgp and MRP expression and response to chemotherapy. Nevertheless, none of the nine Pgp positive patients achieved a complete response whereas 20% did it in the Pgp negative group (not significant). Interestingly, patients with mVP positive tumors had a significantly inferior response to chemotherapy (8% complete, 36% partial, and 56% no response) as compared to patients with mVP negative tumors (50% complete, 30% partial, and 20% no response) ($p= 0.004$ by chi-square test for the three categories of response, and $p= 0.006$ by Fisher's exact test comparing the rates of complete response). When only platinum-treated patients were analyzed the correlation remained significant ($p= 0.01$ in both types of comparisons). When tumors were categorized according to the different combinations of expression of Pgp, MRP, and mVP, only the combination of Pgp and mVP was more significant than mVP alone to predict the rate of complete response (chi-square test, $p= 0.001$).

Expression of Pgp, MRP and mVP and survival

All 57 patients, including those in whom response to chemotherapy was not evaluable, were analyzed for survival. The median progression-free and overall survival for the whole group were 11 and 20 months, respectively; values that fall within the expected range for a group of unselected advanced ovarian carcinoma patients. The univariate survival analysis according to different clinico-pathologic features is summarized in Table 6. No correlation was found between Pgp or MRP expression, and progression-free and overall survival. In contrast, patients with mVP positive tumors had a significant shorter progression-free and overall survival than patients with mVP negative tumors. The median progression-free survival for the mVP positive and mVP negative groups was 9 and 28 months, respectively ($p= 0.003$), and the median overall survival was 15 and 42 months, respectively ($p= 0.007$) (Fig. 2). These correlations remained significant when only the 50

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platinum treated patients were included (p= 0.01, and p= 0.03 for progression-free and overall survival, respectively).

Multivariate analysis of mVP expression, FIGO stage, residual tumor after initial surgery, tumor grade, and presence or absence of ascites, showed that only mVP status was independently related to both progression-free survival and overall survival. No other factor made an additional contribution to the model. After step number one the global chi-square for mVP for progression-free and overall survival was 8.26 (p= 0.004) and 6.81 (p= 0.009), respectively.

DISCUSSION

This study shows that in contrast to Pgp, two newly described MDR-related proteins MRP and mVP are frequently expressed in untreated advanced ovarian carcinoma, denoting their potential as contributors to and/or markers of clinical drug resistance in this tumor. The retrospective analysis of the clinical data demonstrated that only the pretreatment status of mVP was an independent prognostic factor of response to chemotherapy and survival. The lack of independent significance of other known prognostic factors may be due not only to the strong impact of the expression of mVP on prognosis but also to the relatively small number of patients.

The predictive value of mVP seems noticeable as most mVP overexpressing MDR cancer cell lines do not show cross-resistance to platinum or alkylating agents (Scheper RJ, Broxterman HJ, Scheffer GL, et al. Overexpression of a 110 kD vesicular protein in non-P-glycoprotein mediated multidrug-resistance. Cancer Res 53:1475-1479, 1993). Noteworthy, mVP is overexpressed in these cell lines where studied, such as in 8226/MR myeloma cells (Futscher BW, Abbaszadegan MR, Domann F, et al. Analysis of MRP mRNA in mitoxantrone selected, multidrug-resistance human tumor cells. Biochem Pharmacol 47:1601-1604, 1994). Furthermore, in 61 drug-unselected cancer cell lines mVP expression correlated not only with resistance to doxorubicin, but also to carboplatin, cisplatin, and melphalan (Izquierdo MA, Shoemaker RH, Flens MJ, et al, manuscript submitted for publication). These data suggest frequent coexpression of mVP with other resistance genes (i.e. genes involved in resistance to platinum and alkylating agents) resulting in a broad spectrum of drug resistance. This may also be the case in ovarian carcinoma.

Thus, besides potentially contributing to resistance to certain drugs, mVP xpression seems at least a pleiotropic marker of resistance. Alternatively, mVP may be associated with biologically more aggressive tumors, though our clinico-pathological data in ovarian carcinoma do not support this possibility. Further evidence of the prognostic value of mVP through its relation with chemoresistance was obtained in a prospective study including 93 acute myeloid leukemia patients, which showed that mVP was an independent prognostic factor for response to chemotherapy (36).

The molecular characterization of mVP as the human major vault protein has constituted the first step to clarify its actual contribution to drug resistance. Cytoplasmic vaults were originally isolated from vesicle fractions (Kedersha NL, Rome LH. Isolation and characterization of a novel ribonucleoprotein particle: large structures contain a single species of small RNA. J Cell Biol 103:699-709, 1986) and, concordantly, mVP staining is primarily vesicular (Scheper RJ, Broxterman HJ, Scheffer GL, et al. Overexpression of a 110 kD vesicular protein in non-P-glycoprotein mediated multidrug-resistance. Cancer Res 53:1475-1479, 1993). Vaults (» 5%) have also been localized to the nuclear pore complexes (Chugani DC, Rome LH, Kedersha NL. Evidence that vault ribonucleoprotein particles localize to the nuclear pore complex. J Cell Sci 106:23-29, 1993), which control the bidirectional nucleocytoplasmic transport of a wide variety of substrates (Rout MP, Wente SR. Pores for thought: nuclear pore complex proteins. Trends Cell Biol 4:357-365, 1994). Vaults are thought to comprise the transporter units of nuclear pore complexes, thus implicating mVP/vaults in nucleocytoplasmic transporter processes (Rome L, Kedersha N, Chugani D. Unlocking vaults: organelles in search of a function. Trends Cell Biol 1:47-50, 1991; Chugani DC, Rome LH, Kedersha NL. Evidence that vault ribonucleoprotein particles localize to the nuclear pore complex. J Cell Sci 106:23-29, 1993). mVP/vaults may regulate the vesicular and nucleocytoplasmic transport of drugs. In support of this view, entrapping of drugs into exocytotic vesicles and decreased nucleus/cytoplasm ratio were reported in mVP overexpressing MDR cells (Schuurhuis GJ, Broxterman HJ, de Lange JHM, et al. Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. Br J Cancer 64:857-861, 1991; Dietel M, Arps H, Lage H, et al. Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line PG85-257. Cancer Res 50:6100-6106, 1990).

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Lack of correlation between Pgp and MRP expression with response to chemotherapy and survival agrees with the inability of the corresponding genes to confer resistance to cisplatin in transfection experiments (Goldstein LJ, Pastan I, Gottesman, M.M. Multidrug resistance in human cancer. Crit Rev Oncol/Hematol, 12: 243-253, 1992; Grant CE, Valdimarsson G, Hipfner DR, et al. Overexpression of Multidrug Resistance-associated Protein (MRP) increases resistance to natural product drugs. Cancer Res 54:357-361, 1994; Zaman GJR, Flens MJ, van Leusden MR, et al. The human multidrug resistance-associated protein (MRP) is a plasma membrane drug efflux pump. Proc Natl Acad Sci 91:8822-8826, 1994; Cole SPC, Sparks KE, Fraser K, et al. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 54:5902-5910, 1994). But, interestingly, none of the few Pgp positive patients achieved complete remission and the combined study of mVP and Pgp was superior to mVP alone to predict the chance of complete response. The meticulous detection of Pgp may assist in the identification of a subset of particularly resistant tumors. Similarly, MDR1 mRNA expression detected by polymerase chain reaction was found to correlate with response to platinum-based chemotherapy in ovarian carcinoma (Holzmayer TA, Hilsenbeck S, Von Hoff DD, et al. Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas. J Natl Cancer Inst 84:1486-1491, 1992). This apparent relation may be due to MDR1 association with more aggressive tumors or coexpression with other resistance genes (Holzmayer TA, Hilsenbeck S, Von Hoff DD, et al. Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas. J Natl Cancer Inst 84:1486-1491, 1992). However, other studies failed to show such a link (Arao S, Suwa H, Mandai M, et al. Expression of multidrug resistance gene and localization of P-glycoprotein in human primary ovarian cancer. Cancer Res 54:1355-1359, 1994; van der Zee AGJ, Hollema H, Suurmeijer AJH, et al. The value of P-glycoprotein, glutathione S-transferase pi, c-erb-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol, in press, 1995) and since the relation is based on a relatively small number of patients it needs confirmation. Furthermore, the low Pgp expression rate in untreated ovarian carcinoma lessens any potential role in resistance to primary chemotherapy (Arao S, Suwa H, Mandai M, et al. Expression of multidrug resistance gene and localization of P-glycoprotein in human primary ovarian cancer. Cancer Res 54:1355-1359, 1994; van der Zee AGJ, Hollema H, Suurmeijer

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AJH, et al. The value of P-glycoprotein, glutathione S-transferase pi, c-erb-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol, in press, 1995).

For platinum and alkylating agents, GSH-conjugation has been described (Johnson SW, Ozols RF, Hamilton TC. Mechanisms of drug resistance in ovarian cancer. Cancer 71:644-649, 1993). MRP can mediate the transport of certain GSH-conjugates (i.e. leukotriene C_4), and it might cause drug resistance by exporting GSH-drug conjugates out of the cells (Jedlitschky G, Leier I, Buchholz U, et al. ATP-dependent transport of glutathione *S*-conjugates by the multidrug resistance-associated protein. Cancer Res 54:4833-4836, 1994; Müller M, Meijer C, Zaman GJR, et al. Overexpression of the multidrug resistance associated protein (MRP) gene results in increased ATP-dependent glutathione S-conjugate transport. Proc Natl Acad Sci USA, in press, 1994). Our results do not support such a role for MRP in clinical resistance to platinum and alkylating agents in ovarian carcinoma.

In conclusion, in this study the analysis of the expression of the novel MDR-related protein mVP, but not of Pgp and MRP, was a strong and independent prognostic factor to predict tumor response to standard chemotherapy and survival in advanced ovarian carcinoma. When confirmed in a large prospective study, the prognostic information provided by mVP may be incorporated as stratification factor in clinical trials of postoperative chemotherapy and might help selecting patients for alternative therapeutical strategies. The ongoing functional characterization of mVP/vaults will clarify the implication of mVP in a potential novel mechanism of drug resistance. This may lead to innovative approaches to prevent the emergence of drug resistance or reverse the phenotype when it occurs.

EXAMPLE 3

Intrinsic or acquired resistance to antineoplastics limits the effectiveness of conventional treatment for patients with acute myeloid leukemia (AML). Multidrug resistance due to overexpression of the mdr1 gene or its plasma membrane product, P-gp has been implicated as an important cellular mechanism contributing to chemotherapy resistance in this disease (List AF, Multidrug resistance: clinical relevance in acute leukemia. Oncology 1993; 7:23-32). P-gp has been linked to a number of prognostic variables including advanced age, leukemia secondary to an antecedent hematologic disorder or cytotoxic therapy, cytogenetic pattern, and a CD34 surface phenotype (Willman CL,

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Kopecky K, Weick J. et al. Biologic parameters that predict treatment response in the novo acute myeloid leukemia (AML): CD34, but not multidrug resistance (MDR) gene expression, is associated with a decreased complete remission (CR) rate and CD34+ patients less frequently achieve CR with high dose cytosine arabinoside. Proc. Am. Soc. Clin. Oncol. 1992; 11: 262a; List, AF, Spier CM, Cline A et al. Expression of the multidrug resistance gene product (P-glycoprotein) in myelodysplasia is associated with a stem cell phenotype. Br. H. Haematol. 1991; 78: 28-34; Campos L., Guyotat D, Archibald E et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute non-lymphoblastic leukemia cells at diagnosis. Blood 1992; 79: 473-476; Boekhorst PAW, de Leeuw K, Schoester M et al. Predominance of functional multidrug resistance (MDR-1) phenotype in CD34+ acute myeloid leukemia cells. Blood 1993; 82: 3157-3162). Leukemias expressing the P-gp MDR phenotype exhibit lower intracellular drug accumulation and diminished in vitro and in vivo sensitivity to the anthracyclines (Campos L., Guyotat D, Archibald E et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute non-lymphoblastic leukemia cells at diagnosis. Blood 1992; 79: 473-476; Herweijer H, Sonneveld P. Baas F et al. Expression of mdr1 and mdr3 multidrug resistance genes in human acute and chronic leukemias and association with stimulation of drug accumulation by cyclosporin J. Natl. Cancer Inst 1990; 82: 1133-1140; Nooter K, Sonneveld P, Oostrum R, et al. Overexpression of the mdr1 gene in blast cells from patients with acute myelocytic leukemia is associated with stimulation of drug accumulation that can be restored by cyclosporin-A. Int. J. Cancer 1990: 45: 263-268; Pirker R, Waollner J, Geissler K, et al. MDR1 gene expression and treatment outcome in acute myeloid leukemia. J. Natl. Cancer Inst. 1991; 83: 708-712; Marie J-P, Zittoun R, Sikic Bl. Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and in vitro drug sensitivity. Blood 1991; 78: 586-592). This is why agents capable of reversing P-gp-mediated drug resistance in vitro have entered clinical testing as modulators of MDR (S lary E, Caillot D, Chauffert B. et al. Feasibility of using quinine, a potential multidrug resistance-reversing agent, in combination with mitoxantrone and cytarabine for the treatment of acute leukemia. J. Clin. Oncol. 1992, 10: 1730-1736; List AF, Spier C, Greer J, Wolff, et al. Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. J. Clin. Oncol. 1993; 11: 1652-1660).

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However the predictive value of P-gp as an independent marker for treatment failure has been questioned in some studies (Willman CL, Kopecky K, Weick J. et al. Biological parameters that predict treatment response in de novo acute myeloid leukemia (AML): CD34, but not multidrug resistance (MDR) gene expression, is associated with a decreased complete remission (CR) rate and CD34+ patients less frequently achieve CR with high dose cytosine arabinoside. Proc. Am. Soc. Clin. Oncol. 1992; 11: 262a; Ball ED, Lawrence D, Malnar M. et al. Correlation of CD34 and multi-drug resistance P170 with FAB and cytogenetics but not prognosis in acute myeloid leukemia (AML) Blood 1990; 76 (suppl. 1): 252a). The reported incidence of P-gp overexpression in clinically resistant leukemia shows considerable variation, and its correlation with in vitro chemosensitivity has been inconsistant (Ito Y, Tanimoto M. Kumazawa T. et al. Increased P-glycoprotein expression and multidrug-resistant gene (mdr1) amplification are infrequently found in fresh acute leukemia cells: sequential analysis of 15 cases at initial presentation and relapsed stage. Cancer 1989; 63: 1534-1538; Ross DD et al. Enhancement of daunorubicin accumulation, retention, and cytotoxicity by verapamil or cyclosporin-A in blast cells from patients with previously untreated acute myeloid leukemia. Blood 1993; 82: 1288-1299; Marutama Y. et al. Effects of verapamil on the cellular accumulation of daunorubicin in blast cells and on the chemosensitivity of leukemic blast progenitors in acute myelogenous leukemia. Br. J. Haematol. 1989; 72; 357-362; Marie J-P, et al. Daunorubicin uptake by leukemic cells: correlations with treatment outcome and mdr1 expression. Leukemia 1993; 7: 825-831). In a phase I/II trial evaluating cyclosporin-A as a modulator of MDR, we found that in remitting patients mdr1 gene message decreased or was not detected at the time of treatment failure, therefore implicating alternate mechanisms of chemotherapy resistance (List AF et al. Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. J. Clin. Oncol. 1993; 11: 1652-1660).

We have found that an antibody directed against mVP identifies P-gp negative tumor cell lines displaying a classical MDR phenotype characterized by energy-depend nt reduction in cellular drug accumulation that extends to the anthracyclines, mitoxantrone, etoposide and the vinca alkaloids. Unlike P-gp these mVP-positive cell lines are insensitive to chemosensitizers such as verapamil and cyclosporin-A, and antibody staining is restricted to the 110 kD protein currently recognized as being mVP that is overexpressed intracellularly in endomembrane structures. The clinical relevance of this drug r sistance

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phenotype was determined and the frequency of antibody staining and prognostic significance of the immunoreactivity in clinical specimens from patients with AML was evaluated.

METHODS

Patients.

104 specimens from 87 patients with either AML [77 patients] or chronic myeloid leukemia in the myeloid blast phase (CML-BP) [10 patients] presented between January 1992 and December 1993 were evaluated. Sequential specimens obtained prior to treatment and at the time of treatment failure were compared in 17 patients. Diagnostic categories of AML included de novo AML [20 patients], AML arising from an antecedent myelodysplastic syndrome or cytotoxic therapy for a prior malignancy (secondary AML) [26 patients], and leukemia in relapse from conventional therapy [31 patients]. Prior treatment with an anthracycline or mitoxantrone was recorded and summarized in Table 8. Response to intensive remission induction therapy was assessed in 73 patients with AML receiving one of four chemotherapy regimens (Table 8). Treatment differed according to age, risk category and prior therapy. Patients with de novo AML received conventional-dose cytarabine (AC 100 mg/m^2/day x 7) administered with daunorubicin (DNR 45 mg/m^2/day x 3) or idarubicin (Ida 12 mg/m^2/day x 3); patients with high-risk AML received either high-dose cytarabine (HiDAC 3 g/m^2/day x 5) administered sequentially with DNR and cyclosporin-A (Sandimmune; Sandoz, East Hanover, NJ) as previously described (List AF et al. Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. J. Clin. Oncol. 1993; 11: 1652-1660), HiDAC plus DNR or mitoxantrone without cyclosporine, or mitoxantrone (mitox 10 mg/m^2/day x 5) and etoposide (VP16 100 mg/m^2/day x 5). Four patients were not evaluatable including three elderly patients with secondary AML who refused induction therapy and one patient with relapsed leukemia who underwent allogen ic bone marrow transplantation.

Response Assessment.

Response to treatment was assessed after one or two courses of conventional-dose induction chemotherapy, or after one course of a HiDAC containing regimen. A partial response

required normal peripheral blood counts with a marrow aspirate containing greater than 5%, but less than 25% blasts; or a marrow aspirate with less than 5% blasts in the presence of moderate thrombocytopenia (50,000 to 100,000/ml platelest) in a patient with secondary AML.

Cytogenetics.

Cytogenetic analysis was performed on bone marrow aspirates from patients with previously untreated AML by routine Giemsa-banding techniques following 24-hour culture and methotrexate synchronization as previously described (Rowley JD, Potter D. Chromosome banding patterns in acute nonlymphocytic leukemia. Blood 1979; 47: 705-721. Chromosome abnormalities were analyzed separately for relationship to Mab staining, and according to prognostic groups using a modification of the Chicago classification in which a normal karyotype, t (Pirker R, Wallner J, Geissler K, et al. MDR1 gene expression and treatment outcome in acute myeloid leukemia. J. Natl. Cancer Inst. 1991; 83: 708-712; Rowley JD, Potter D. Chromosome banding patterns in acute nonlymphocytic leukemia. Blood 1979; 47: 705-721), abnormal 16, t (Marutama Y. et al. Effects of verapamil on the cellular accumulation of daunorubicin in blast cells and on the chemosensitivity of leukemic blast progenitors in acute myelogenous leukemia. Br. J. Haematol. 1989; 72; 357-362; Chen Y-N, et al. Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. J. Biol. Chem. 1990; 265: 10073-10080) and abnormal 11q23 were considered favorable, and other chromosom abnormalities and complex patterns, unfavorable (Samuals BL. et al. Specific chromosomal abnormalities in acute nonlymphocytic leukemia correlate with drug susceptibility in vitro. Leukemia 1988; 2: 79-83).

Immunocytochemistry.

Mononuclear cell fractions were isolated from heparinized bone marrow aspirates by double Ficoll-Hypaque density gradient centrifugation. Cytospins were prepared and blast reactivity with mVP (R.J. Scheper, Amsterdam, the Netherlands) and PgP-specific antibodies were determined by indirect immunoperoxidase using a previously described modified biotin-avidin technique (Scheper RJ, et al. Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 1993; 53: 1475-1479;

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Grogan T. et al. Optimization of immunocytochemical P-glycoprotein assessment in multidrug resistant plasma cell myeloma using three antibodies. lab. Invest. 1990; 63: 815-824). Sensitive parental (8226/s) and two multidrug resistant human myeloma cell lines selected with either mitoxantrone for non-Pgp MDR (MR20) or doxorubicin for classical MDR (Dox6) served as negative, mVP-positive, or Pgp-positive controls, respectively. Samples were reported as positive when staining was detected in 20% or more of the blast fraction.

Cytofluorography.

Surface marker analysis was performed by flow cytometry using monoclonal antibodies directed against Pgp (MRK16), $CD34^+$, CD7 (Becton-Dickinson, Mountain View, CA) and myeloid specific antigens (List AF et al. Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. J. Clin. Oncol. 1993; 11: 1652-1660. Cell suspensions were incubated with monoclonal antibodies for 30 minutes at 4°C, washed, then incubated with fluorescein-isothiocyanate (FTIC) conjugated goat anti-mouse immunoglobulin. Cells were washed and resuspended in buffer containing 0.5% paraformaldehyde fixative. Indirect immunofluorescence was analyzed by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). A nonreactive murine antibody of the same isotype served as control. Test samples were considered positive when labelling was detected in 20% or more of the gated cell population relative to the control.

Statistical Analysis.

Response rates and other univariate analyses were compared by the Fisher's exact test. Progression-free and overall survival was estimated with the methods of Kaplan and Meier (Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J. Am. Stat. Asso 1958; 53: 457-481). Comparisons between subgroups were performed by log-rank analysis (Mantel N. Evaluation of survival data and two new rank order statistics arizing in its consideration. Cancer Chemother. Rep. 1966; 50: 163-170).

RESULTS.

Immunocytochemical staining with mVP was localized primarily to the cytoplasm in a granular fashion (Figure 6). Overall, 34 leukemia specimens showed immunoreactivity in 20% or more of the blast population. mVP reactivity was detected in all disease categories, ranging in frequency from 10% in patients with CML-BP, to 50% in secondary AML (Table 9). In patients with AML, mVP immunoreactivity was associated with an inferior response to induction chemotherapy (complete and partial remission, 36% versus 65%; P = 0.003) owing to a higher prevalence of resistant disease in mVP-positive patients (Table 10). When analyzed according to mVP and P-gp staining pattern, immunoreactivity with either marker was not mutually exclusive, and each marker identified patients at greater risk for induction failure (Table 11). Patients with dual staining had the lowest remission rate (31%) and a corresponding higher prevalence of resistant disease (58%) suggesting an additive prognostic effect, although a statistically significant difference could not be discerned because of limited sample size.

The probability of progression-free survival in the mVP-negative and mVP-positive patients is shown in Figure 7. Progression-free survival in patients lacking mVP immunoreactivity was significantly longer than the mVP-positive cohort (P= 0.006). In a linear regression analysis of outcome according to mVP and P-gp phenotype, P-gp retained prognostic relevance for response to induction therapy (P = 0.009), but mVP was an independent prognostic marker for progression-free survival (P=0.0015).

Characteristics of treatment that might select for the mVP phenotype ware analyzed in the relapsed patient cohort. Prior treatment with mitoxantrone was associated with a significantly higher probability of mVP reactivity compared to patients receiving daunorubicin or idarubicin (P=0.004) (Table 8). Among 17 patients receiving treatment with daunorubicin and cyclosporin-A, sequential samples obtained prior to treatment and at the time of disease progression showed acquisition of the mVP phenotype despite a loss or decrease in frequency of Pgp expression (P=0.02) (Table 12). This effect was most apparent in patients who achieved a complete remission with the cyclosporin-based regimen.

The relationship between clinical and biological prognostic variables and mVP is summarized in Table 7. Significant correlations in AML patients were found with Pgp expression (P= 0.007), a CD7 surface phenotype (P= 0.008), and age greater 55 years (P=0.012) in patients with AML. No association was found with a CD34 surface phenotype or cytogenetic pattern. The latter may relate to the small number of previously untreated

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patients available for analysis. Only 22 of the 46 patients with de novo or secondary AML had an abnormal karyotype. Although there was no association with specific chromosomal abnormalities, mVP-positivity was noted in 3 of 5 samples from patients with structural or numerical deletions of chromosome 7, but in none of the 6 patients with the favorable translocations t (Pirker R, Wallner J, Geissler K, et al. MDR1 gene expression and treatment outcome in acute myeloid leukemia. J. Natl. Cancer Inst. 1991; 83: 708-712; Rowley JD, Potter D. Chromosome banding patterns in acute nonlymphocytic leukemia. Blood 1979; 47: 705-721) [N=2] and t (Marutama Y. et al. Effects of verapamil on the cellular accumulation of daunorubicin in blast cells and on the chemosensitivity of leukemic blast progenitors in acute myelogenous leukemia. Br. J. Haematol. 1989; 72; 357-362; Chen Y-N, et al. Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. J. Biol. Chem. 1990; 265: 10073-10080) [N=4].

DISCUSSION

Chemotherapy resistance may result from any of a number of biological mechanisms. In this prospective study, we found that the MDR phenotype recognized by a monoclonal antibody against mVP has prognostic importance in AML. mVP immunoreactivity was detected in 39% of patients specimens, including both previously treated and untreated patients [Table 9]. Like Pgp, the highest prevalence was detected in patients with secondary AML (50%). Although the frequency of mVP detection in the relapsed patient cohort was comparable to that for P-gp (42% versus 45%), there was preferential selection for this alternate MDR phenotype by treatment with the anthracenedione, mitoxanthrone [Table 7]. This observation is consistent with results of selection studies performed in tumor cell lines. Although mVP was raised against the doxorubicin-selected, non-small cell lung cancer cell line SW-1573, the anthracyclines as a group more often select for Pgp, whereas mitoxantrone selection favors the development of alternate MDR mechanisms (Scheper RJ, et al. Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 1993; 53: 1475-1479; Harker WG et al. Multidrug resistance in mitoxanthrone-selected HL-60 leukemia cells in the absence of P-glycoprotein overexpression. Cancer Res. 1989; 49: 4542-4549;

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Taylor CW, et al. Different mechanisms of decreased drug accumulation in doxorubicin and mitoxanthrone resistant variants of the MCF7 human breast cancer cell line. Brit J. Cancer 1991; 63: 923-929; Nakagawa M. et al. Reduced intracellular drug accumulation in the absence of P-glycoprotein (mdr1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells. Cancer Res. 1992; 52: 6175-6181).

Pretreatment detection of the mVP phenotype was predictive of success of induction chemotherapy, as evidenced by the superior rate of remission and progression-free survival in mVP-negative patients. This difference in treatment outcome resulted from a higher frequency of resistant failure in mVP-positive patients [Table 10]. Because P-gp overexpression was common in mVP-positive specimens, stratification of outcome according to drug resistance phenotype was analyzed to determine the relative contribution of each marker. As shown in Table 11, each drug resistance marker adversely influenced response to induction therapy, but only mVP retained prognostic significance for progressive-free survival in linear regression analysis [P=0.015]. It is possible that a prognostic effect of P-gp may have been obscured by treatment with the P-gp modulator cyclosporin-A in many patients. However, the prognostic significance of each marker appeared additive, such that the highest rate of induction failure was noted in patients with dual staining. If confirmed in a larger series, pretreatment screening of patients specimens for each of these MDR phenotypes might provide a reliable means to identify patients at particularly high risk for treatment failure with conventional therapy, who therefore might be considered for alternate treatment strategies.

Previous investigations have shown that P-gp overexpression in AML is principally restricted to those leukemias which display a CD34+ or stem cell surface phenotype, a pattern mimicking its regulation in normal hematopoiesis (Willman CL, Kopecky K, Weick J. et al. Biologic parameters that predict treatment response in de novo acute myeloid leukemia (AML): CD34, but not multidrug resistance (MDR) gene expression, is associated with a decreased complete remission (CR) rate and CD34+ patients less frequently achieve CR with high dose cytosine arabinoside. Proc. Am. Soc. Clin. Oncol. 1992; 11: 262a; List, AF, Spier CM, Cline A et al. Expression of the multidrug resistance gene product (P-glycoprotein) in myelodysplasia is associated with a stem cell phenotype. Br. H. Haematol. 1991; 78: 28-34; Campos L., Guyotat D, Archibald E et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute non-lymphoblastic

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leukemia cells at diagnosis. Blood 1992; 79: 473-476; Boekhorst PAW, de Leeuw K, Schoester M et al. Predominance of functional multidrug resistance (MDR-1) phenotype in CD34+ acute myeloid leukemia cells. Blood 1993; 82: 3157-3162); Chaudhary PM Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hemapoietic stem cells. Cell 1991; Cell 1991; 66: 85-94; Drach D et al. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype/ Blood 1992; 80: 2729-2734).

Although there was no correlation between CD34 and mVP in the present study, the finding that mVP is closely associated with CD7 indicates linkage of this drug resistance marker to a primitive stem cell phenotype [Table 7]. While CD34 is an antigenic marker for b th multipotential and lineage-committed progenitors (Chaudhary PM Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hemapoietic stem cells. Cell 1991; Cell 1991; 66: 85-94; Drach D et al. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype/ Blood 1992; 80: 2729-2734), CD7 expression in the absence of mature T-cell antigens identifies pluripotent stem cells capable of lymphoid and/or myeloid differentiation (Kurtzberg J. et al. Immature human thymocytes can be driven to differentiate into nonlymphoid lineages by cytokines from thymic epithelial cells. Proc. Natl. Acad. Sci USA 1989; 86: 7575-7579;Barcena A, et al. Phenotypic and functional analysis of T-cell precursors in the human fetal liver and thymus: CD7 expression in the early stages of T- and myeloid-cell development. Blood 1993; 82: 3401-3414; Kurtzberg J. et al. CD7+, $CD4^-$. $CD8^-$ acute leukemia: A syndrome of malignant pluripotent lymphohematopoietic cells. Blood 1989; 73: 381-390). Recent reports that CD7 identifies a phenotypic AML cohort with a particularly poor prognosis suggest that the drug resistance phenotype defined by mVP may contribute to the adverse biological behavior observed in this group of patients (Kita K. et al. (The Japanese Cooperative Group of Leukemia/Lymphoma). Clinical importance of CD7 expression in acute myelocytic leukemia. Blood 1993; 81: 2399-2405; Schwarzinger I, et al. Prognostic significance of surface marker expression on blasts of patients with de novo acute myeloblastic leukemia. J. Clin. Oncol. 1990; 8: 423-430; Del poetyer G. et al. CD7 expression in acute myeloid leukemia. Blood 1993; 82: 2929-2930). These findings raise important questions regarding the physiological relevance of this drug resistant phenotype. Analogous to P-gp, the molecule recognized by mVP is highly expressed in many normal tissues including bronchial,

intestinal and renal epithelium, but not bile canalucili (Scheper RJ, et al. Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 1993; 53: 1475-1479). Expression is also found in peripheral blood lymphocytes. In the SW-1573 cell line, this alternate MDR phenotype precedes emergence of P-gp, the latter of which requires greater drug selection pressure (Scheper RJ, et al. Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 1993; 53: 1475-1479; Schuurhuis GJ, et al. Early multidrug resistance defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. Brit J. Cancer 1991; 64: 857-861). Our finding that mVP is perhaps associated with an earlier stem cell phenotype than P-gp suggests that this pattern of regulation may be conserved from normal progenitors and warrants further investigation.

mVP immunoreactivity was associated with other adverse prognostic variables, including advanced age and P-gp, but no correlation was found with cytogenetic pattern [Table 7]. The latter finding may be explained by the relatively small number of previously untreated patients in the analysis. Nevertheless, antibody staining was not detected in specimens from the 6 patients with favorable chromosome translocations, whereas 3 of 5 patients with deletions of chromosome 7 were mVP-positive, suggesting that a relationship with cytogenetic pattern might be demonstrable in a larger series of patients.

Treatment with a chemotherapy regimen incorporating cyclosporin-A as a modulator of P-gp function selected for mVP expression despite a decrease or loss of P-gp (Table 12). This observation confirms that antibody directed against mVP may identify leukemic clones distinct from those harboring P-gp, and is in agreement with results of in vitro selection studies. Myeloma cell lines selected for resistance to both doxorubicin and the P-gp modulator verapamil exhibit mVP overexpression, but lack P-gp (W.S. Salton, unpublished data). Our findings that mVP overexpression has independent prognostic reliance in AML indicated that cellular mechanisms other than P-gp contribute to anthracycline resistance in this disease. The relatively high prevalence of mVP in AML and its apparent selection by cyclosporin-A suggest that this drug resistance phenotype may limit the effectiveness of MDR1 modulators in clinical trials. The latter is under investigation in an ongoing Southwest Oncology Group (SWOG) trial in which participants are randomized to an anthracycline-containing induction regimen with or without the addition of cyclosporin-A. In the study of Example 2 in patients with ovarian cancer, mVP overexpression was found to

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confer a lower response rate to cisplatin-based chemotherapy, indicating that the prognostic value of mVP extends to other malignancies (Rik, Scheper, unpublished data).

EXAMPLE 4

The complementary DNA coding for the mVP gene product was isolated by expression cloning using mouse MOP8 cells and the LRP 56 monoclonal antibody to screen a cDNA library derived from the human multidrug resistant (MDR) fibrosarcoma cell line HT108 DR4 (Slovak, ML et al. Cancer Res. 48, 2793-2797 (1988). Confirmation of the isolation of the full length cDNA was obtained by ^{35}S-immunoprecipitation. From both control MDR tumor cell lines and mVP transfected MOP8 cells the predicted MR 110 KD protein was specifically precipitated. The sequence displays a single open reading frame f 2688 base pairs coding for an 896 amino acid protein with the calculated MR of a 100 KD. Scanning the mVP amino acid sequence for cites and signatures on the PC gene program Prosite® (Bairoch, A., Nucleic Acid Res. 20, 2013-2018 (1992)) failed to provide functional clues. Notably there was no indication of transmembrane fragments and the sequence did not contain the ATP-binding active transport signature, characteristic for the transmembrane transporter protein p-glycoprotein and MRP. A computer assisted search of the Swiss protein bank showed strong homology with the *Dictyostelium discoideum* major vault protein a which accounts for more than 70% of the composition of vaults. Alignment of the two protein sequences showed 57,3% of the amino acids are identical. Levels of the cognate DNA could now be detected with the RN-ase protection assay. In SW1573 and GLC4 MDR suppliance RNA levels were detected in accordance with protein levels.

METHODS.

Expression cloning was performed as previously described (Wijngaard, P.L.J., Metzelaar, M.J., McHugh, N.D., Morrison, W.I. and Clevers, H.C. J. Immunol. 149, 3273-3277; Aruffo, A. and Seed, B. Proc. Natl. Acad. Sci. 84, 8573 (1987).

* Preparation of c-DNA sublibraries. Poly(A)+RNA was isolated from the non P-gp MDR subline HT1080 Dr4 using RNAzol and an oligo dT column. A size-fractionated (>2000 bp) oligo dT primed c-DNA library was constructed in a pCDM8 vector using non-self complementary BstXI linkers. High voltage electroporation (25 uF, 200 ohm, 2.5 kV/cm) of the library into the *Escherichia coli* strain MC1061/p3 yielded

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approximately 100,000 primary colonies. These were divided into 10 sublibraries of 10,000 colonies each.

* Isolation of the mVP c-DNA clone. Trypsinised MOP8 cells ($5x10^6$/sample) were transfected for 45 minutes at 37°C in 2 ml RPMI 1640/2% Nu serum (Becton Dickinson, Bedford, USA) containing 250 mg/ml DEAE-dextran and 1 mg/ml of purified plasmid from the sublibraries. The cells were washed once and cultured in Dulbecco's modified Eagle's medium, containing 10% FCS and antibiotics for 48 h. Thereafter, cells were trypsinised, spun down on octo-cytospin slides, dried, acetone fixed for 5 minutes and immunostained with LRP-56 MAb (2 mg/ml, in PBS/1% BSA), biotinylated rabbit-anti-mouse (1:150 in PBS/1% BSA, Zymed, San Francisco, CA, USA) and streptavidin-horse radish peroxidase (1:500 in PBS/1% BSA, Zymed, San Francisco, CA, USA). Positive cells were visualised with 0.02% 9-amino-3-ethyl-carbazole/0.1% hydrogen peroxide in 0.1 M sodium acetate, pH 4.8. The mVP c-DNA was isolated from one of the sublibraries by screening progressively smaller pools of bacterial colonies. The mVP nucleotide sequence is available at the EMBL, GenBank and Nucleotide Sequence databases under accession number X 79882.

^{35}S-methionine precipitation with LRP-56 MAb.

MOP8 cells were transfected with irrelevant or mVP containing pCDM8 plasmids, as described. Precipitation was carried out as described previously (Scheper, R.J., Broxterman, H.J., Scheffer, G.L., Kaaijk P., Dalton, W.S., van Heijningen, T.H.M., van Kalken, C.K., Slovak. M.L., de Vries, E.G.E., van der Valk, P., Meijer, C.J.L.M., and Pinedo, H.M. Cancer Res. 53, 1475-1479 (1993)). MOP8 cells, 24 h after transfection, and MDR control cells were incubated for 2 h at 37°C in methionine free culture medium without FCS. Cells were incubated for a further period of 16 h at 37°C after addition of 10% FCS containing ^{35}S-methionine giving a final concentration of 4 m Ci/ml. Cells were trypsinised and lysed in PBS containing 1% NP40, 1 mM EDTA, 1 mM PMSF for 20 minutes at room temperature. Large fragments and nuclei were removed by centrifugation at 9000 x g. Equal cpm's (25 x 10^6/sample) of clear supernatants were incubated for 30 minutes with protein A-sepharose beads to reduce non-specific binding of labelled protein to the beads. supernatants were incubated with 15 mg of LRP-56 MAb or 15 mg of irrelevant MAb (data not shown) for 4 h at 4°C. Then 50 ml of prot A-sepharose beads (~ 25 ml 'packed beads') was added and

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precipitation was allowed for 1 h. Precipitates were washed 3x in lysis buffer/1% milk powder/0.01% SDS and 3 x PBS. Antibody-antigen interaction was broken by dispensing the beads in loading buffer, containing 200 mM Tris-HCl (pH 6.8), 1% ß-mercaptoethanol, 8% SDS, 10% glycerol and 0.05% bromophenol blue. Supernatants were loaded for overnight 4-12% gradient PAAGE after which the gel was fixed for 30 minutes in 10% acetic acid, 20% methanol. Signals were amplified with NAMP 100 fluid (Amersham, Little Chalfont, U.K.) and the gel was dried. Precipitated protein was visualised by autoradiography.

Figure legends

Figure 1.

Correlation of the MDR rank (as an average relative resistance to MDR related drugs) and non-MDR rank (as an average relative resistance to MDR unrelated drugs) among a series of drug unselected cell lines comprising the NCI panel used for anti-cancer drug screening. R, Spearman's rank correlation coefficient. Each mark represents one cell line.

Figure 2.

Relative frequency (%) of MDR-associated proteins according to staining index groups. P-gp data are given for comparison (P-gp was considered positive if any of the three MAbs used was positive). MRP and mVP were positive in 87% and 78% of the cell lines, respectively (P-gp 24%). The expression of MRP and mVP (and P-gp) showed a positive skewed distribution with most cell lines displaying low to moderate expression. The level of expression was lower than that of the MDR cell lines GLC4/ADR and SW-1573/2R120 used as a MRP and mVP control, respectively. The corresponding parental cell lines GLC4 and SW-1573 were classified in the lowest group of expression.

Figure 3.

Correlation between MDR rank and mVP and MRP expression. A cut-off staining index (SI) of 0,60 was selected to differentiate "low" from "high" mVP and MRP expressing cell lines. The group of cell lines with high mVP expression (SI > 0,60) showed higher MDR rank (___ mean rank ± SD, 36 ± 11,8) as compared to cell lines with a low mVP expression (SI ≤ 0,60; 26 ± 13,8) (t test, P = 0,004). No difference was observed between low and high MRP expressing cell lines (27 ±, and 32 ±, respectively; t test). Each mark represents one cell line.

Figure 4.

The cell lines of the panel were divided into three equally large groups according to the MDR rank: low, moderate and high MDR rank, respectively. Each group was compared for mVP and MRP expression. Cell lines with low MDR rank had a significantly lower mVP expression (mean staying index ± SD, 0,28 ± 0,48) as compared to the intermediate (1,19 ± 0,84) and high (0,91 ± 0,78) groups (Welch test, F 10,45, P = 0,0003). In contrast, a

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complete overlap was observed with regard to MRP expression between the three groups as some lines with low MDR had high MRP expression (Welch test, F =1,00, P = 0,38). Each mark represents one cell line.

Figure 5.

Progression-free survival according to LRP56 phenotype in 73 patients with AML receiving intensive induction chemotherapy.

* Sequence id. no. 1 = EMBL Genbank accession number X79882.

* Sequence id. no. 2 = amino acid sequence corresponding to human mVP as deposited at the EMBL Genbank accession number X79882.

The following pages illustrate the results of alignments of known mVP sequences and the novel mVP sequence id no. 2. mVP is indicated as mVP, the rat protein is indicated as RT.

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The deduced amino acid sequence of the mVP protein alligned with the *Dictyostelium discoideum* major Vault protein α.

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LRP        - MATEEFIIRIPPYHYIHVLDQNSNVSRVEVGPKTYIRQDNERVLFAPMRM -50
mVp Dictyo- MADLNSVIRIKPFHFIHVLDNNTNVTRVEVGPQTFTRQDHEKLVSGPEPM -50

LRP        - VTVPPRHYCTVANPVSRDAQGLVLFDVTGQVRLRHADLEIRLAQDPFPLY -100
mVp Dictyo- IMIPQRNYCTISNPVVRNENGSLVLDEYGQVKLRHGDEEIRFSQEPFPLY -100

LRP        - PGEVLEKDITPLQVVLPNTALHLKAL----------LDFEDKDGDKVVA -139
mVp Dictyo- PGEKISGKVTALQVVAPLKALRLRALRRDFTEAKVTH----------A -138

LRP        - GDEWLFEGPGTYIPRKEVEVVEIIQATIIRQNQALRLRARKECWDRDGKE -189
mVp Dictyo- GDEWLFEGPATYLPRIDVRIEEEIKATIIGPNQALKLRANKACSDRSGVA -188

LRP        - RVTGEEWLVTTVGAYLPAVFEEVLDLVDAVILTEKTALHLRARRNFRD-F -238
mVp Dictyo- RKAGEEWLVRQHGAYLPGNDEKVVEIVNAYVLTDKKALHLKATKTFLDET -238

LRP        - RGVSRRTGEEWLVTVQDTEAHVPDVHEEVLGVVPITTLGPHNYCVILDPV -288
mVp Dictyo- RKTQRKAGEEWLVVSTDAETHIPDVYEQVVGEVHITTLSNRQYCVVLDPI -288

LRP        - GPDGKNQLGQKRVVKGEKSFFLQPGEQLEQG-IQDVYVLSEQQGLLLRAL -337
mVp Dictyo- GANGKPQLGHKQLRKGELAFFLNPGESLEGNKIHNIYVLTEQEALLLRA- -337

LRP        - QPLEEGEDEEKVS-----HQAGDHWLIRGPLEYVPSAKVEVVEERQAIPL -382
mVp Dictyo- --------KETFSIDGESHLAGDRWMIYGPCDYVPPVQVEVVEKRESIPL -379

LRP        - DENEGIYVQDVKTGKVRAVIGSTYMLTQDEVLWEKELPPGVEELLNKGQD -432
mVp Dictyo- DENEGIYVRDIKTGKVASIKGQSYMLKANEELWEKVLPRTVEEVLAKE-- -427

LRP        - PLADRGEKDTAKSLQPLAP-----RNKTRVVSYRVPHNAAVQVYDYREKR -477
mVp Dictyo- ---------------SLNPEFTDNRDSTRVVTYRAPHNSAVQIYDYKEKK -462

LRP        - ARVVFGPELVSLGPEEQFTVLSLSAGRPKRPHARRALCLLLGPDFFTDVI -527
mVp Dictyo- SRVVFGPDLVMLGPDEHFTVLSLSGDKPKRPHQIKAIALFFGPDFMTDVV -512

LRP        - TIETADHARLQLQLAYNWHFEVNDRKDPQETAKLFSVPDFVGDACKAIAS -577
mVp Dictyo- IVETSDHARLSLKLSYNWEFKV-DRSKIEDAQKIFQVPDFVGDSCKAIAS -561

LRP        - RVRGAVASVTFDDFHKNSARIIRTAVFGFETSE---------AKGPDGMA -618
mVp Dictyo- RVRGAVAAVSFDDFHKRSAEVIRQSVFGLDESGEVRKNFSFNS------- -604

LRP        - LPRPRDQAVFPQNGLVVSSVDVQSVEPVDQRTRDALQRSVQLAIEITTNS -668
mVp Dictyo- ----------NNLVITNIDIQSVEPVDQRTRDSLQKSVQLAIEITTKS -642

LRP        - QEAAAKHEAQRLEQEARGRLERQKILDQSEAEKARKELLELEALSMAVES -718
mVp Dictyo- QEAAARHEAERLEQGARGRLERQKIHDEAQAELARKDLLQLQAQSAAVES -692
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LRP         - TGTAKAEAESRAEAARIEGEGSVLQAKLKAQALAIETEAELQRVQKVREL -768
              ||  |  |||  .  ||||  |   |   |  ||  |||||   |  .|.|.   .    ||
mVp Dictyo- TGQATAEAQATAEAANIAAEANVKQAELKAQATKIRSESEISLLKAKREN -742

LRP         - ELVYARAQLELEVSKAQQLAEVEVKKFKQMTEAIGPSTIRDLAVAGPEMQ -818
              ||  |  ..   |||..|    |||.|   |||  .  |.||   |..  .|  ||  |||
mVp Dictyo- ELSYQKSIDELELTKQSDLAEIEASKFKAIVESIGRDTLKSIACAGNEMQ -792

LRP         - VKLLQSLGLKSTLITDGSTPINLFNTAFGLLG--------MGPEGQPLGRR -861
               ||||  |||||  .||||  .|.|||  ||  |..|          |          |||
mVp Dictyo- AKLLQGLGLKSFMITDGKSPLNLFDTANGIIGNNNQMVNMSNAAKQSGRR -842

LRP         - VPVAQPWGGDIPPVCSGPSSSWRQPRGACTALTPD -896

mVp Dictyo- ----------------------------------N -843

Identity  : 483 ( 57.3%)  Similarity: 111 ( 13.2%)
```

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===05-FEB-1995=========================================================PC/GENE===

**************************************
* ALIGNMENT OF TWO PROTEIN SEQUENCES. *
**************************************

The two sequences to be aligned are:

 mVP
Total number of residues: 896.

 RT
Total number of residues: 895.

Comparison matrix   : Structure-genetic matrix.
Open gap cost       : 10
Unit gap cost       : 2

The character to show that two aligned residues are identical is '|'
The character to show that two aligned residues are similar is '.'
Amino acids said to be 'similar' are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W

mVP    - M----------ATEEFIIRIPPYHYIHVLDQNSNVSRVEVGPKTYIRQDNERVL -44
RT     - MFGGKSLGFTMATEEAIIRIPPYHYIHVLDQNSNVSRVEVGPKTYIRQDNERVL -54

mVP    - FAPMRMVTVPPRHYCTVANPVSRDAQGLVLFDVTGQVRLRHADLEIRLAQDPFP -98
RT     - FAPVRMVTVPPRHYCIVANPVSRDTQSSVLFDITGQVRLRHADQEIRLAQDPFP -108

mVP    - LYPGEVLEKDITPLQVVLPNTALHLKALLDFEDKDGDKVVAGDEWLFEGPGTYI -152
RT     - LYPGEVLEKDITPLQVVLPNTALHLKALLDFEDKNGDKVMAGDEWLFEGPGTYI -162

mVP    - PRKEVEVVEIIQATIIRQNQALRLRARKECWDRDGKERVTGEEWLVTTVGAYLP -206
RT     - PQKEVEVVEIIQATVIKQNQALRLRARKECFDREGKGRVTGEEWLVRSVGAYLP -216

mVP    - AVFEEVLDLVDAVILTEKTALHLRARRNFRDFRGVSRRTGEEWLVTVQDTEAHV -260
RT     - AVFEEVLDLVDAVILTEKTALHLRALQNFRDLRGVLHRTGEEWLVTVQDTEAHV -270

mVP    - PDVHEEVLGVVPITTLGPHNYCVILDPVGPDGKNQLGQKRVVKGEKSFFLQPGE -314
RT     - PDVYEEVLGVVPITTLGPRHYCVILDPMGPDGKNQLGQKRVVKGEKSFFLQPGE -324

mVP    - QLEQGIQDVYVLSEQQGLLLRALQPLEEGEDEEKVSHQAGDHWLIRGPLEYVPS -368
RT     - RLERGIQDVYVLSEQQGLLLKALQPLEEGESEEKVSHQAGDCWLIRGPLEYVPS -378

mVP    - AKVEVVEERQAIPLDENEGIYVQDVKTGKVRAVIGSTYMLTQDEVLWEKELPPG -422
RT     - AKVEVVEERQAIPLDQNEGIYVQDVKTGKVRAVICSTYMLTQDEVLWEKELPSG -432

mVP    - VEELLNKGQDPLADRGEKDTAKSLQPLAPRNKTRVVSYRVPHNAAVQVYDYREK -476
RT     - VEELLNLGHDPLADRGQKGTAKPLQPSAPRNKTRVVSYRVPHNAAVQVYDYRAK -486

mVP    - RARVVFGPELVSLGPEEQFTVLSLSAGRPKRPHARRALCLLLGPDFFTDVITIE -530
RT     - RARVVFGPELVTLDPEEQFTVLSLSAGRPKRPHARRALCLLLGPDFFTDVITIE -540

mVP    - TADHARLQLQLAYNWHFEVNDRKDPQETAKLFSVPDFVGDACKAIASRVRGAVA -584
RT     - TADHARLQLQLAYNWHFELKNRNDPAEAAKLFSVPDFVGDACKAIASRVRGAVA -594
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mVP   - DVQSVEPVDQRTRDALQRSVQLAIEITTNSQEAAAKHEAQRLEQEARGRLERQK -692
RT    - DVQSVEPVDQRTRDALQRSVQLAIEITTNSQEAAAKHEAQRLEQEARGRLERQK -702

mVP   - ILDQSEAEKARKELLELEALSMAVESTGTAKAEAESRAEAARIEGEGSVLQAKL -746
RT    - ILDQSEAEKARKELLELEAMSMAVESTGNAKAEAESRAEAARIEGEGSVLQAKL -756

mVP   - KAQALAIETEAELQRVQKVRELELVYARAQLELEVSKAQQLAEVEVKKFKQMTE -800
RT    - KAQALAIETEAELERVKKVREMELIYARAQLELEVSKAQQLANVEAKKFKEMTE -810

mVP   - AIGPSTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSTPINLFNTAFGLLGMGPE -854
RT    - ALGPGTIRDLAVAGPEMQVKLLQSLGLKSTLITDGSSPMNLFSTASGCWGWGLM -864

mVP   - GQPLGRRVPVAQPWGGDIPPVCSGPSSSWRQPRGACTALTPD -896
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Identity : 785 (87.7%)
Similarity: 23 (2.6%)
Number of gaps inserted in mVP : 1
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Akzo Nobel N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM

(ii) TITLE OF INVENTION: A method for identifying a novel multidrug-resistance type.

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2840 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GCCCCCATGC GCATGGTGAC CGTCCCCCCA CGTCACTACT GCACAGTGGC CAACCCTGTG 300

TCTCGGGATG CCCAGGGCTT GGTGCTGTTT GATGTCACAG GGCAAGTTCG GCTTCGCCAC 360

GCTGACCTCG AGATCCGGCT GGCCCAGGAC CCCTTCCCCC TGTACCCAGG GGAGGTGCTG 420

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 896 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Arg Met Val Thr Val Pro Pro Arg His Tyr Cys Thr Val Ala Asn Pro
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Val Ser Arg Asp Ala Gln Gly Leu Val Leu Phe Asp Val Thr Gly Gln
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Phe Pro Leu Tyr Pro Gly Glu Val Leu Glu Lys Asp Ile Thr Pro Leu
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Phe Glu Asp Lys Asp Gly Asp Lys Val Val Ala Gly Asp Glu Trp Leu
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Table 1

Non-P-glycoprotein mdr cell lines which exhibit a defect in drug accumulation

Isolate	Drug used for selection[a]	Parental cell line	Verapamil reversal of resistance[b]	Reference[c]
HL60/AR	ADR(100)	human leukemia,promyelocyte	+	1
HL60/ADR	ADR(10)	human leukemia, promyelocyte	+	2
COR-L23/R	ADR(17)	human large cell lung	+	3
GLC4/R	ADR(32)	human small cell lung	ND	4
SK-MEL-170/R	ADR(178)	human melanoma	ND	5
H69/AR[c]	ADR(32)	human small cell lung	-	6
HT1080/R	ADR(129)	human fibrosarcoma	+	7
SW1573/120	ADR(10)	human squamous lung	-	8
PC4/40	ADR(40)	murine erythroleukemia	+	9
WiDr/R	Mitox(21)	human colon	-	10
MCF-7/Mitox	Mitox(120)	human breast	-	11
MCF-7/MX	Mitox(4000)	human breast	-	12
KB/40a	VP16 (287)	human nasopharyngeal	+	13
G5	MGBG(40)	Adenovirus transformed rat brain cells	-	14
LALW-2	Therapy induced	human leukenic T-cells	ND	15

[a] Drug designations: ADR - Adriamycin; Mitox - Mitoxantrone; MGBG - methylglyoxalbis-(quanylhydrazone). Numbers in parentheses refer to level of resistance to the selecting agent.

[b] Reversal of resistance by verapamil is indicated by a + sign. The relative extent of resistance reversal varies, however, with different cell lines (see text). A + indicates that verapamil is capable of inducing an increase in drug accumulation and/or reducing drug IC_{50} values. A -sign indicates that verapamil has no effect on resistance levels. ND = effect of verapamil on resistance was not determined.

[c] Recent studies indicate that H69/AR cellsoverexpress a protein (MRP) which has sequence homology with P-gp and other proteins which may be involved in transport processes (Cole et al., 1992). Although H69/AR cells are not defective in drug accumulation (Cole et al., 1991) in MRP protein may function to partition drug away from its cytotoxic target. This sequestration process may be operative in other non-P-gp mdr cell lines.

[d] 1= Bhalla et al. (1985) Isolation and characterization of an anthracycline resistant human leukemic cell line. Cancer Res. 45: 3657-3662

2= Marsh et al. (1986) Isolation and characterization of adriamycin-resistant HL60 cells which are not defective in the initial intracellular accumulation of drug. Cancer Res. 46: 4053-4057 of Dimethylsulfoxide, retinoic acid and 12-O-tetradecanoylphorbol-13-acetate induce a selective decrease in the phosphorylation of P150, a surface membrane phosphoprotein of HL60 cells resistant to adriamycin. Biochem. Biophys. Res. Commun. 138: 9-16.

3= Twentyman et al. (1986) Derivation and preliminary characterization of adriamycin resistant lines of human lung cancer cells. Br. J. Cancer 53: 529-537.

4= Zijlstra et al. (1987) Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res. 47: 1780-1784.

5= Panneerselvan et al.

6= Mirski et al. (1987) Multidrug resistance in a human small cell lung cancer cell line selected in Adriamycin. Cancer Res. 47: 2594-2598.

7= Slovak et al. (1988) Pharmacol. and biol. evidence for differing mechanisms of doxorubicin resistance in two human cell lines. Cancer Res. 48: 2793-2797.

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8= Keizer et al. (1989) Correlation of multidrug resistance with decreased drug accumulation, altered subcellular drug distribution, and increased P-glycoprotein expression in cultured SW-1573 human lung tumor cells. Cancer Res. 49: 2988-2993.

9= Slapak et al. (1990) Sequential emergence of distinct resistance phenotypes in murine erythro leukemic cells under adriamycin selection: decreased anthracycline uptake precedes increased P-glycoprotein expression. Cancer Res. 50: 7895-7901.

10= Dalton et al. (1988) Cytogenetic and phenotypic analysis of a human colon carcinoma cell line resistant to mitoxantrone. Cancer Res. 48: 1882-1888.

11= Taylor et al. (1991) Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF-7 human breast cancer cell line. Br. J. Cancer 63: 923-929.

12= Nakagawa et al. (1992) Reduced intracellular drug accumulation in the absence of P-glycoprotein (mdr1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells. Cancer Res. 52: 6175-6181.

13= Ferguson et al. (1988) Combined modalities of resistance in etoposide-resistant human KB cell lines. Cancer Res. 48: 5956-5964.

14= Weber et al. (1989) Non-P-glycoprotein-mediated multidrug resistance in detransformed rat cells selected for resistance to methylglyoxal bis(quanylhydrazone). Cancer Res. 49: 5779-5783.

15= Haber et al. (1989) Atypical multidrug resistance in a therapy-induced drug resistant human leukemia cell line (LALW-2): resistance to vinca alkaloids independent of P- glycoprotein. 'Cancer Res. 49: 5281-5287.

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Table 2 - Cellular parameters relevant to multidrug resistance

Cell line[a]	Tumor type	MDR rank[f]	Non-MDR rank[e]	Staining index[c]		
				PGP	MRP	mVP
OVCAR-4	Ovary	56.71	39.27	0.22[d]	1.80	1.00
EKVX	Lung	56.57	56.60	0.45	0.05	1.65
TK-10	Renal	54.07	51.67	0.00	1.20	1.60
UO-31	Renal	53.71	37.53	0.18	1.70	2.40
NCI-H322M	Lung	53.00	46.43	0.00	ND	ND
HCT-15	Colon	52.00	36.40	1.01	1.40	0.10
OVCAR-5	Ovary	51.57	41.73	0.00	0.10	1.40
HOP-18	Lung	50.43	42.29	0.00	ND	1.50
SNB-78	CNS	50.43	46.67	0.00	0.80	1.30
DLD-1	Colon	49.71	38.87	0.59	0.00	0.15
SK-MEL-28	Melanoma	46.86	46.40	0.00	0.70	0.20
SK-0V-3	Ovary	44.14	44.70	0.00	0.55	0.60
RXF-393	Renal	43.71	30.53	0.00	0.65	0.30
UACC-257	Melanoma	43.29	39.80	0.00	0.65	0.01
OVCAR-3	Ovary	42.71	36.80	0.00	0.20	0.00
IGROV1	Ovary	42.29	35.00	0.00	0.30	1.80
HCC-2998	Colon	41.86	36.53	0.00	1.10	1.95

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(Table 2)

Cell line[a]	Tumor type	MDR rank[f]	Non-MDR rank[e]	Staining index[c]		
				PGP	MRP	mVP
SK-MEL-2	Melanoma	41.14	50.67	0.00	0,15	0.05
A498	Renal	41.00	46.47	0.00	ND	ND
KM20L2	Colon	36.43	40.93	0.00	0.80	1.50
M19-MEL	Melanoma	36.00	41.20	0.00	1.70	0.60
KM12	Colon	35.14	48.20	0.00	0.40	0.00
RXF-631	Renal	34.64	31.07	0.00	0.05	1.20
SNB-75	CNS	34.57	32.73	0.00	0.40	0.60
COLO-205	Colon	34.50	39.33	0.31	1.75	2.70
M14	Melanoma	34.29	25.33	0.00	0.85	1.40
SF-268	CNS	33.00	16.13	0.00	0.70	0.00
HT29	Colon	32.71	41.40	0.00	0.00	1.00
HOP-92	Lung	32.29	27.60	0.00	ND	0.20
OVCAR-8	Ovary	30.71	34.00	0.07	1.20	1.40
CAKI-1	Renal	30.29	18.47	0.47	0.35	2.25
ACHN	Renal	29.71	20.93	0.45	0.00	1.50
MALME-3M	Melanoma	29.14	34.27	0.00	0.75	1.60
SNB-19	CNS	28.00	38.80	0.00	0.00	0.00
LXFL-529L	Lung	27.57	46.33	0.00	0.75	1.80

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(Table 2)

Cell line[a]	Tumor type	MDR rank[f]	Non-MDR rank[g]	Staining index[c]		
				PGP	MRP	mVP
K-562	Leukemia	26.86	33.27	ND	0.70	0.00
SF-295	CNS	26.43	21.67	0.00	1.50	0.80
HOP-62	Lung	26.29	25.53	0.00	1.50	1.70
HCT-116	Colon	25.29	28.50	0.15	1.80	2.55
NCI-H23	Lung	25.29	23.20	0.00	0.30	0.15
NCI-H226	Lung	23.29	43.40	0.62	0.80	1.50
	Renal	22.86	28.87	0.00	0.40	1.50
	Melanoma	22.71	30.73	0.00	1.60	1.60
SN12	CNS	21.43	14.87	0.00	0.20	0.60
SK-MEL-5	Lung	20.57	25.13	0.00	0.00	0.00
SF-539	Lung	20.43	24.60	0.00	0.10	0.00
A549	Renal	20.14	16.20	0.09	0.45	1.50
NCI-H522	Melanoma	20.00	11.33	0.00	1.25	0.02
786-O	colon	18.71	33.27	0.08	0.60	0.30
UACC-62	SCLC	18.71	21.27	0.00	0.70	0.25
SW-620	Leukemia	15.86	10.33	ND	0.40	0.06
DMS-114	CNS	14.71	27.00	0.00	0.85	0.10
HL60	Leukemia	14.57	29.40	ND	0.00	0.50
U251						
RPMI 8226						

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(Table 2)

Cell line[a]	Tumor type	MDR rank[f]	Non-MDR rank[e]	Staining index[c]		
				PGP	MRP	mVP
XF-498	CNS	13.43	33.47	0.00	ND	0.30
CEM	Leukemia	13.14	12.13	ND	0.85	0.00
MCF-7	Breast	11.71	19.07	0.00	0.50	0.00
DMS-273	SCLC	10.07	14.20	0.00	0.30	0.00
LOX	Melanoma	10.00	14.20	0.00	0.80	0.00
NCI-H460	Lung	8.71	10.07	0.00	0.40	0.15
MOLT-4	Leukemia	8.43	9.73	ND	0.00	0.00
SR	Leukemia	4.86	6.87	ND	0.90	0.00

[a] Cell lines are in decreasing order of MDR rank; [c] Staining index was calculated as the product of % positive cells and staining intensity as described in "Materials and Methods"; [d]The highest SI of any of the 3 monoclonal antibodies (JSB-1, MRK-16, C219) used is stated (14); [e] MDR unrelated drugs tested: thioguanine, S-mercaptopurine, 5-fluorouracil, 5-FUDR, hydroxyurea, 1-ß-D-arabinofuranosylcytosine, busulfan, nitrogen mustard, chlorambucil, melphalan, CCNU, cis-platinum, CBDCA, BCNU, and bleomycin; [f] MDR related drugs tested: actinomycin D, vinblastine sulfate, vincristine sulfate, daunomycin, adramycin, VP-16, and AMSA. Ranks were calculated as described in "Materials and Methods".

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Table 3

Correlation between drug sensitivity and expression of MDR-associated proteins

	Staining index Pgp[a]		Staining index MRP[b]		Staining index mVP	
	Spearman's correlation	t test (£0,15vs>0,15)[c]	Spearman's correlation	t test (£1.1vs>1.1)[c]	Spearman's correlation	t test (£0.6vs>0.6)[g]
Global ranks[d]						
MDR rank	0.17 (ns)[e]	0.01	0.12 (ns)	ns	0.40 (0.002)	0.004
Non-MDR rank	0.05 (ns)	ns	0.11 (ns)	ns	0.36 (0.005)	0.01
Individual drugs[e]						
*Doxorubicin	0.32 (0.02)	0.01	0.18 (ns)	ns	0.27 (0.04)	0.03
*Vincristine	0.14 (ns)	ns	0.37 (0.004)	0.04	0.37 (0.004)	0.02
*VP-16	0.08 (ns)	ns	0.18 (ns)	ns	0.22 (ns)	ns
*Mitoxantrone	0.09 (ns)	ns	0,12 (ns)	ns	0.21 (ns)	ns
**Cisplatin	0.14 (ns)	ns	0.13 (ns)	ns	0.28 (0.05)	ns
**Carboplatin	0.29 (0.03)	ns	0.12 (ns)	ns	0.39 (0.002)	0.01
**Melphalan	0.001 (ns)	ns	0.02 (ns)	ns	0.28 (0.03)	0.02

[a] The highest staining index of any of the 3 monoclonal antibodies (JSB-1, MRK-16, C219) was used (14). [b] According to the staining index obtained with MAb MRP..1: equivalent results were found with MAB MRPm6. [c] The more significant cut-off staining index for each MDR associated protein was used. [d] For calculation of MDR and non-MDR ranks see "Materials and Methods". [e] Spearman's rank correlation coefficient (p value): ns not significant (p>0.05). [f] p value of the t test. [g] Correlation with log (GI_{50}).

Table 4. Patient characteristics of 57 advanced ovarian carcinoma patients treated with chemotherapy according to Pgp, MRP, and mVP expression*.

	Pgp expression		MRP expression		mVP expression	
	Negative	Positive	Negative	Positive	Negative	Positive
Number of patients	48 (84)†	9 (16)	18 (32)	39 (68)	13 (23)	44 (77)
Age (years)						
≤ 60 (n=22)	19 (40)††	3 (33)	7 (39)	15 (38)	4 (31)	18 (41)
> 60 (n=35)	29 (60)	6 (67)	11 (61)	24 (62)	9 (69)	26 (59)
Performance status‡						
0/1 (n=20)	15 (68)	5 (83)	4 (57)	16 (76)	4 (57)	16 (76)
2/3 (n=8)	7 (32)	1 (17)	3 (43)	5 (24)	3 (43)	5 (24)
FIGO stage						
III (n= 43)	36 (75)	7 (78)	12 (67)	31 (79)	9 (69)	34 (77)
IV (n= 14)	12 (25)	2 (22)	6 (33)	8 (21)	4 (31)	10 (23)
Residual tumor						
≤ 2 cm (n=24)	19 (40)	5 (56)	6 (33)	18 (46)	7 (54)	17 (39)
> 2 cm (n=33)	29 (60)	4 (44)	12 (67)	21 (54)	6 (46)	27 (61)
Histology						
Serous (n=40)	33 (69)	7 (78)	12 (67)	28 (72)	8 (62)	32 (73)
Mucinous (n=5)	4 (8)	1 (11)	2 (11)	3 (8)	0	5 (11)
Undi. car.§(n=8)	8 (17)	0	3 (17)	5 (12)	3 (23)	5 (11)
Endometrioid (n=4)	3 (6)	1 (11)	1 (5)	3 (8)	2 (15)	2 (5)
Grade						
I/II (n=21)	17 (35)	4 (44)	7 (39)	14 (36)	2 (15)	19 (43)
III (n=36)	31 (65)	5 (56)	11 (61)	25 (64)	11 (85)	25 (57)
Ascites						
Present (n=37)	32 (67)	5 (56)	12 (67)	25 (64)	7 (54)	30 (68)
Absent (n= 20)	16 (33)	4 (44)	6 (33)	14 (36)	6 (46)	14 (32)
Chemotherapy						
Platinum-based (n= 50)	41 (85)	9 (100)	17 (94)	33 (85)	12 (92)	38 (86)
Cis/Car + Cy‖	38	9	16	31	12	35
Cis + Car	3	0	1	2	0	3
Alkylating (n=7)	7 (15)	0	1 (6)	6 (15)	1 (8)	6 (14)
Melphalan	6	0	0	6	1	5
Chlorambucil	1	0	1	0	0	1

* All the comparisons (chi-square or Fisher's exact test) between Pgp, MRP, and mVP expression groups with the different variables were statistically not significant ($p > 0.05$).

† Number of patients (percentage of the total).

†† Number of patients (percentage within each expression group).

‡ Data available for 28 patients.

§ Undifferentiated carcin ma.

‖ Cis, cisplatin; Car, carboplatin; Cy, cyclophosphamide. Including an anthracycline in: four Pgp negativ and on Pgp p sitive patients, two MRP negative and three MRP positive patients, and five mVP positive patients.

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Table 5. Relation between Pgp, MRP and mVP expression and response to chemotherapy (n = 49)*

		Complete response	Partial response	No response	p value
Pgp	negative (n = 40)	8 (20%)	13 (32%)	19 (48%)	0.33†
	positive (n = 9)	0	4 (44%)	5 (56%)	0.32‡
MRP	negative (n = 16)	3 (19%)	5 (31%)	8 (50%)	0.69†
	positive (n = 33)	5 (15%)	12 (36%)	16 (49%)	0.67‡
mVP	negative (n = 10)	5 (50%)	3 (30%)	2 (20%)	0.004†
	positive (n = 39)	3 (8%)	14 (36%)	22 (56%)	0.006‡

*Eight out of 57 patients were not evaluable for response (eight in the Pgp negative group, two and six in MRP negative and MRP positive groups, and three and five in mVP negative and mVP positive groups).

† By chi-square test comparing the three categories of response.

‡ By Fisher's exact test comparing the rate of complete response.

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Table 6. Univariate analysis of clinicopathologic paramet rs and multidrug resistance-associated proteins in 57 patients with advanced ovarian carcinoma*

	Progression free	Overall survival
Age (years)	p = 0.87†	p = 0.54
≤ 60 (n = 22)	10 ± 4.56‡	26 ± 8.24
> 60 (n = 35)	11 ± 1.33	15 ± 3.64
FIGO stage	p = 0.05	p = 0.03
III (n = 43)	11 ± 2.03	22 ± 4.96
IV (n = 14)	5 ± 0.94	8 ± 0.94
Residual tumor	p = 0.07	p = 0.04
≤ 2 cm (n = 24)	15 ± 2.94	30 ± 4.67
> 2 cm (n = 33)	8 ± 2.05	12 ± 2.67
Histology	p = 0.92	p = 0.94
Serous (n = 40)	11 ± 1.16	20 ± 6.98
Others (n = 17)	11 ± 5.49	18 ± 4.12
Grade	p = 0.18	p = 0.02
I/II (n = 21)	16 ± 3.56	27 ± 9.86
III (n = 36)	9 ± 2.21	12 ± 2.17
Ascites	p = 0.04	p = 0.06
Present (n = 37)	16 ± 3.56	15 ± 6.42
Absent (n = 20)	18 ± 3.76	30 ± 8.40
Pgp	p = 0.77	p = 0.70
Negative (n = 48)	11 ± 2.71	12 ± 10.30
Positive (n = 9)	11 ± 4.98	20 ± 3.77
MRP	p = 0.45	p = 0.67
Negative (n = 18)	11 ± 5.56	15 ± 1.67
Positive (n = 39)	11 ± 1.19	20 ± 5.34
mVP	p = 0.003	p = 0.007
Negative (n = 13)	28 ± 7.5	42§
Positive (n = 44)	9 ± 2.41	15 ± 3.07

* Performance status is not include since data for only 28 patients were available.

† p value of the log-rank test.

‡ Data are median survival in months ± asymptotic standard error.

§ The asymptotic standard error could not be c mputed becaus the 25th quantile was not reach d.

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Table 7

Clinical and Biological Features Associated with the mVP Phenotype		
Characteristic	Percent mVP-Positive	P-value*
Prior mitoxantrone*		0.0122
mitox + other°	63	
DNR or Ida only	17	
CD7 Surface phenotype		0.0421
CD7-positive	65	
CD7-negative	33	
Pgp expression		0.047
Pgp-positive	53	
Pgp-negative	30	
Age^		0.0169
age > 55 years	57	
age ≤ 55 years	20	
CD34 surface phenotype		0.814
CD34-positive	41	
CD34-negative	45	
Cytogenetics^		0.556
normal	46	
abnormal	35	

* Relapsed patients only

° Denotes prior treatment with mitoxantrone alone or in addition to other anthracycline

^ *De novo* and secondary AML

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Table 8

Patient Demographics and Treatm nt	
	Number
Median age (years)	52
(range)	(13-84)
Sex (M:F)	58:35
Diagnosis	
AML	83
de novo	21
secondary	27
relapsed	35
1st relapse	*14*
2nd or greater	*16*
primary refractory	*5*
CML-BP	10
Prior anthracycline treatment* (Relapsed AML)	
mitoxantrone ± other	16
DNR or Ida	18
Chemotherapy induction regimens (AML)	
AC (7) + DNR (3)	24
HiDAC + DNR/CsA°	36
HiDAC + DNR or Mitox	6
Mitox + VP16	3

* Includes mitoxantrone

° CsA denotes cyclosporin-A

Table 9

mVP Immunoreactivity and P-glycoprotein Expression By Disease Category			
Diagnosis	No. Patients	mVP+ (%)	Pgp+ (%)
Acute myeloid leukemia (AML)			
De novo	21	7 (33)	8 (38)
Secondary	27	13 (48)	17 (63)
Relapsed	35	14 (40)	15 (43)
CML-BP	10	1 (10)	5 (50)
Total	93	35 (38)	45 (48)

Table 10

Remission Rate in Patients with AML According to mVP Phenotype		
	mVP-Negative	mVP-Positive
Number of patients	41	28
Complete or partial remission	28 (68)*	9 (32)°
Resistant disease	6 (15)	18 (57)
Early deaths	7	3

* Values in parentheses represent percentage

° P = 0.0008

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Table 11

Treatment Outcome According to MDR Phenotype				
mVP/Pgp Phenotype	No.	Complete or Partial Remission	Resistant Failure	Early Death
mVP & Pgp-negative	25	19 (76)*	2 (8)	4
mVP-negative/Pgp-positive	16	9 (56)	4 (25)	2
mVP-positive/Pgp-negative	11	4 (36)	6 (36)	1
mVP & Pgp-positive	17	4 (29)	12 (59)	2

* Values in parentheses represent percentage

Table 12

Response Category	No. of patients	Pre-Treatment		Progression	
		Pgp+	mVP+	Pgp+	mVP+
Resistant disease	8	6	2	5	3
Relapse from Complete remission	9	5	0	1	7
Total	17	11	2(12)	6	10(59)*

* P = 0.034; represents comparison of mVP reactivity pretreatment vs time of progression

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Table 13

Cytogenetic Pattern	(N=47)*	mVP-Positive (%)
Normal	24	11(46)
Abnormal	23	8 (35)
Favorable	8	1 (12)
t (15;17)	4	0
t (8;21)	3	0
inv (16)	15	1
Unfavorable	15	7 (47)
Trisomy 8	1	1
-7/7q-	3	2
-5/5q-	2	0
Both -7/7q- & -5/5q-	3	1
Other	6	3

* Includes only previously untreated patients with *de novo* or secondary AML

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CLAIMS

1. A method for identifying a multi drug resistant cell exhibiting multi drug resistance of a novel type to be called VR-MDR, said method comprising determining the presence of a nucleic acid sequence encoding a vault component and/or determining the presence of at least one vault-component said component being a protein or RNA sequence.

2. A method according to claim 1 wherein the presence of the major protein component of a human vault is determined.

3. A method according to claim 2, wherein the amino acid sequence of the protein shows at least 60% homology with the sequence of sequence ID No.2.

4. Method according to any of claims 1-3, wherein an antibody is used capable of binding to a protein component of a vault.

5. A method according to claim 1, wherein the presence and optionally the amount of a nucleic acid sequence is determined in the cell, said nucleic acid sequence either encoding a protein component of the human vault or said nucleic acid sequence being the RNA sequence comprised in the vault.

6. A method for inhibiting VR-MDR type drug resistance of a cell, comprising inhibiting the production and/or the activity of a protein, said protein being a vault-component.

7. A method according to claim 6 wherein the production of the protein is inhibited by down regulating initiation of expression of the protein.

8. A method according to claim 6 wherein the production of the protein is inhibited by a) contacting the cell with a molecule that comprises at least part of the antisense nucleic acid sequence complementary to the nucleic acid sequence encoding the

protein, or by

b) contacting the cell with a ribozyme targeted to the nucleic acid sequence encoding the protein, in an amount sufficient to inhibit the production of the protein.

9. A method according to claim 6 wherein the activity of the protein is inhibited by contacting the protein with an antibody specific for an epitope on the protein.

10. A method according to any of claims 6-10, wherein the protein is the major protein component of a human vault.

11. A method for protecting drug sensitive cells from cytotoxicity due to exposure to a drug for which VR-MDR type occurs, said method comprising increasing the amount or activity of vaults by specifically transfecting the cell with at least one nucleic acid sequence being or encoding a component of a human vault, or a functional equivalent of said component(s), said nucleic acid sequence or cell comprising regulatory elements enabling production of the component(s) or the equivalent(s) thereof by the cell.

12. A nucleic acid sequence encoding at least part of the amino acid sequence of the major protein component of a human vault or a functional equivalent thereof.

13. A nucleic acid sequence being capable of hybridizing under normal to stringent hybridizing conditions to at least part of the nucleic acid sequence of Sequence ID NO. 1 or to at least part of the complementary sequence thereof.

14. A recombinant vector comprising a nucleic acid sequence according to claim 12 or 13 operatively linked to a regulatory sequence.

15. A transformant host cell including a recombinant vector according to claim 15.

16. A monoclonal antibody specific for an epitope on a protein component of a human vault with the provisio that the Mab is not MabLRP56.

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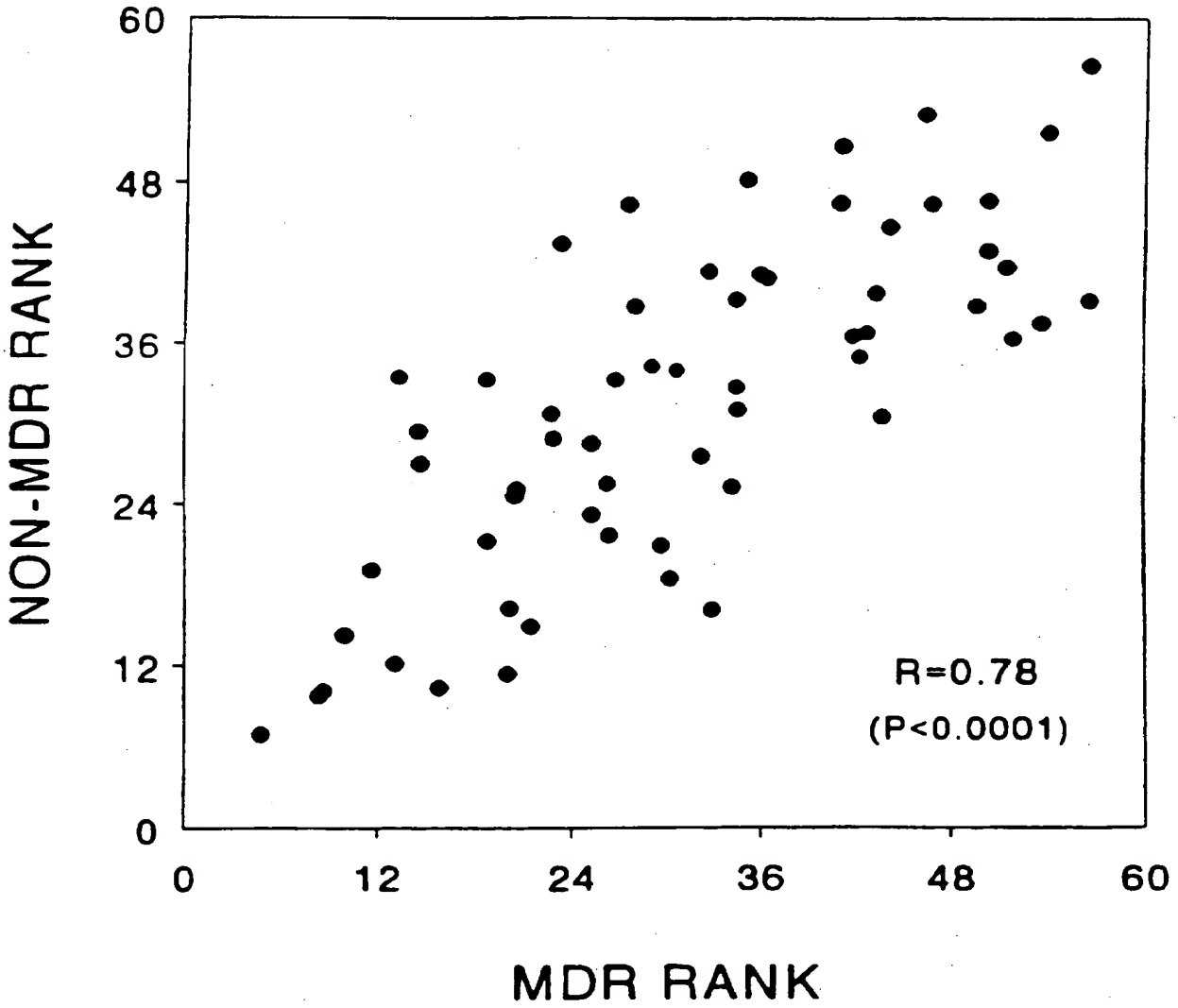
17. An antibody specific for an epitope on a protein component of a human vault, coupled to a substance having toxic or therapeutic activity whilst retaining the ability to bind to the epitope.

18. A diagnostic kit comprising an antibody specific for an epitope on a protein component of a human vault, coupled to a solid phase or a labeling substance and means for detecting any immune complexes formed between the antibody and the protein.

19. A test-system comprising a first cell or organism said test system further comprising a second cell said second cell being of the corresponding type as the first cell differing only in the expression of less or none of the vault-proteins, such that said first cell exhibits VR-MDR and the second cell either exhibits MDR of a non-VR-MDR type or no MDR at all.

Figures 1/5

fig-1



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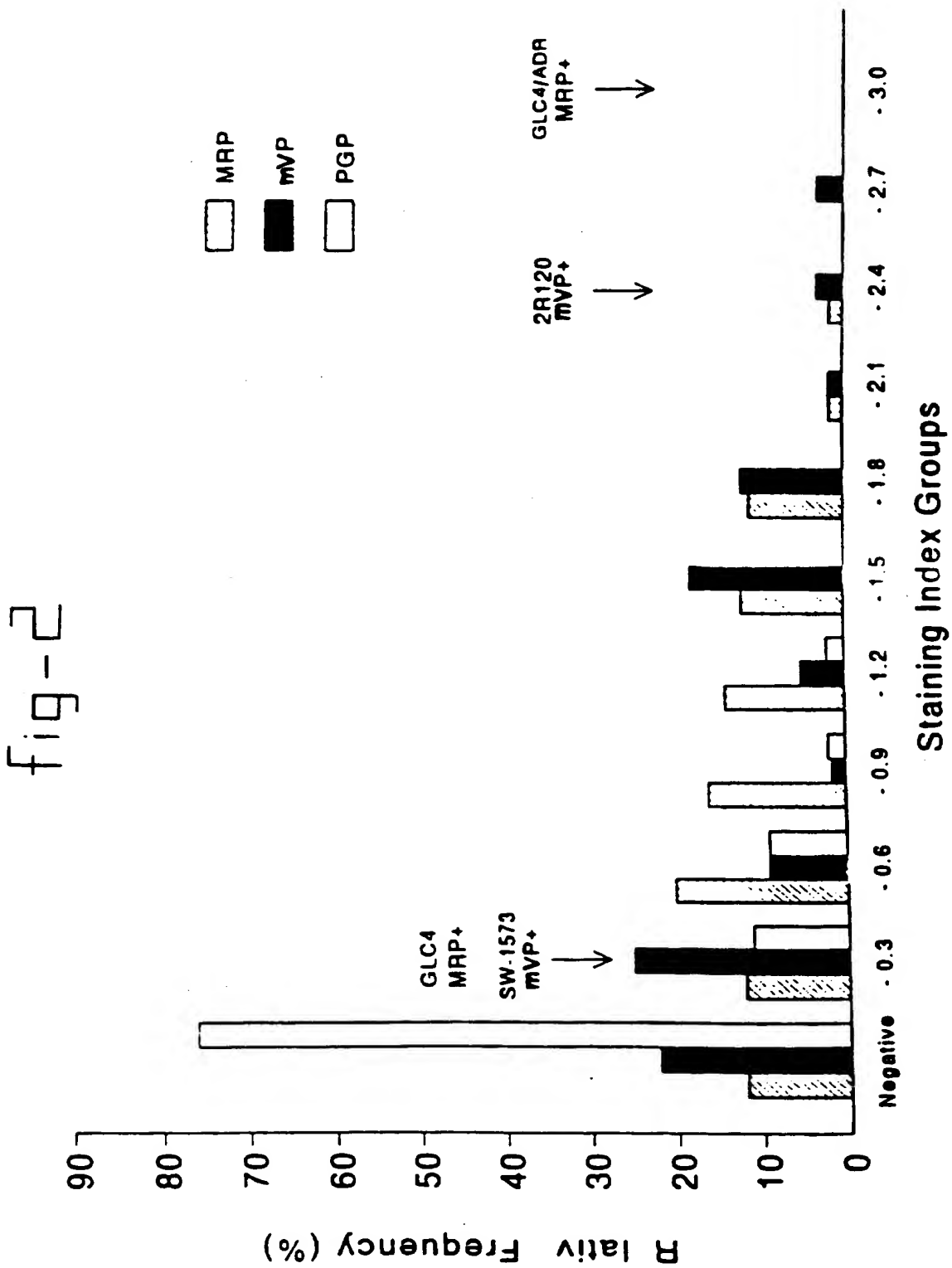

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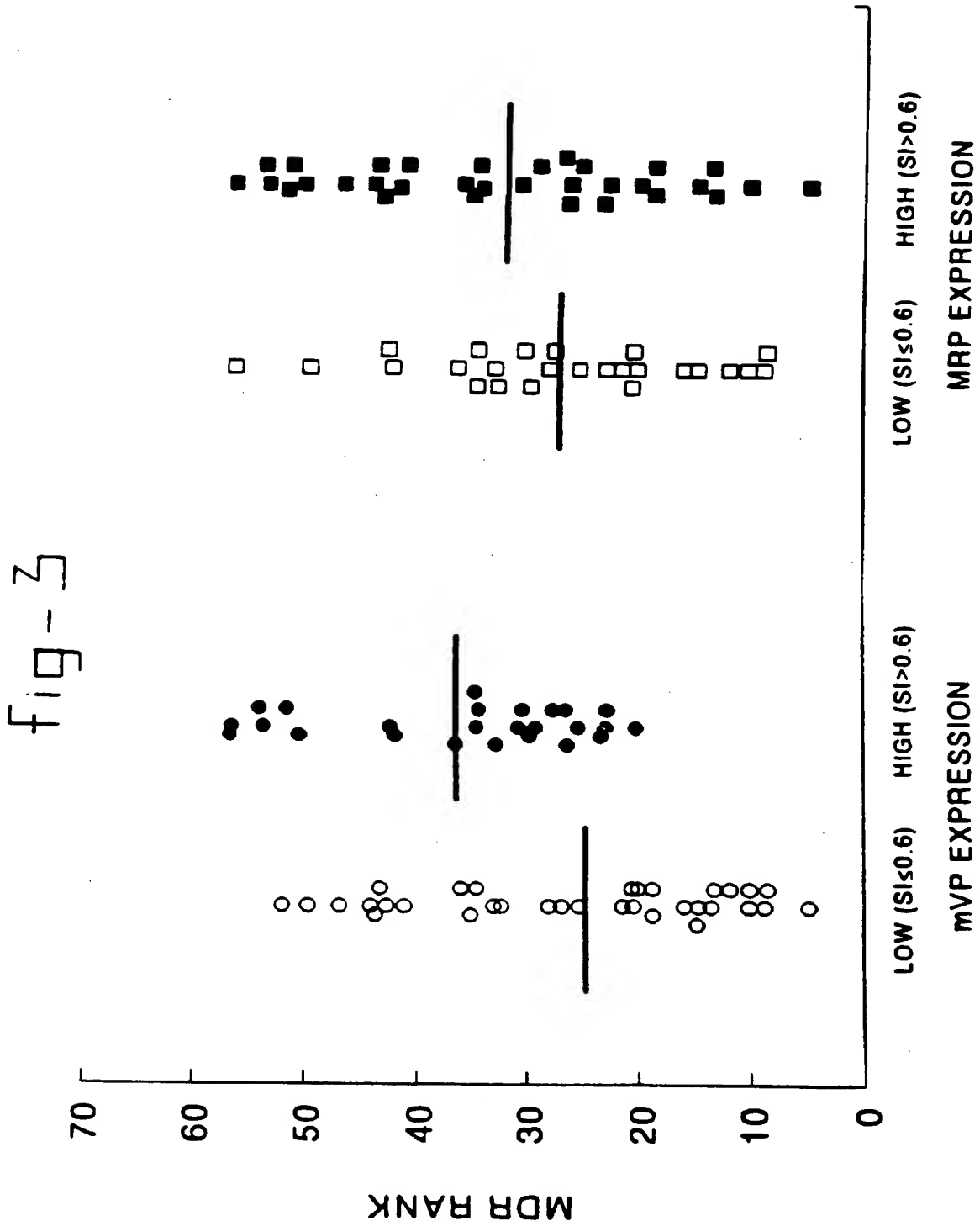
Figures 3/5



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Figures 4/5

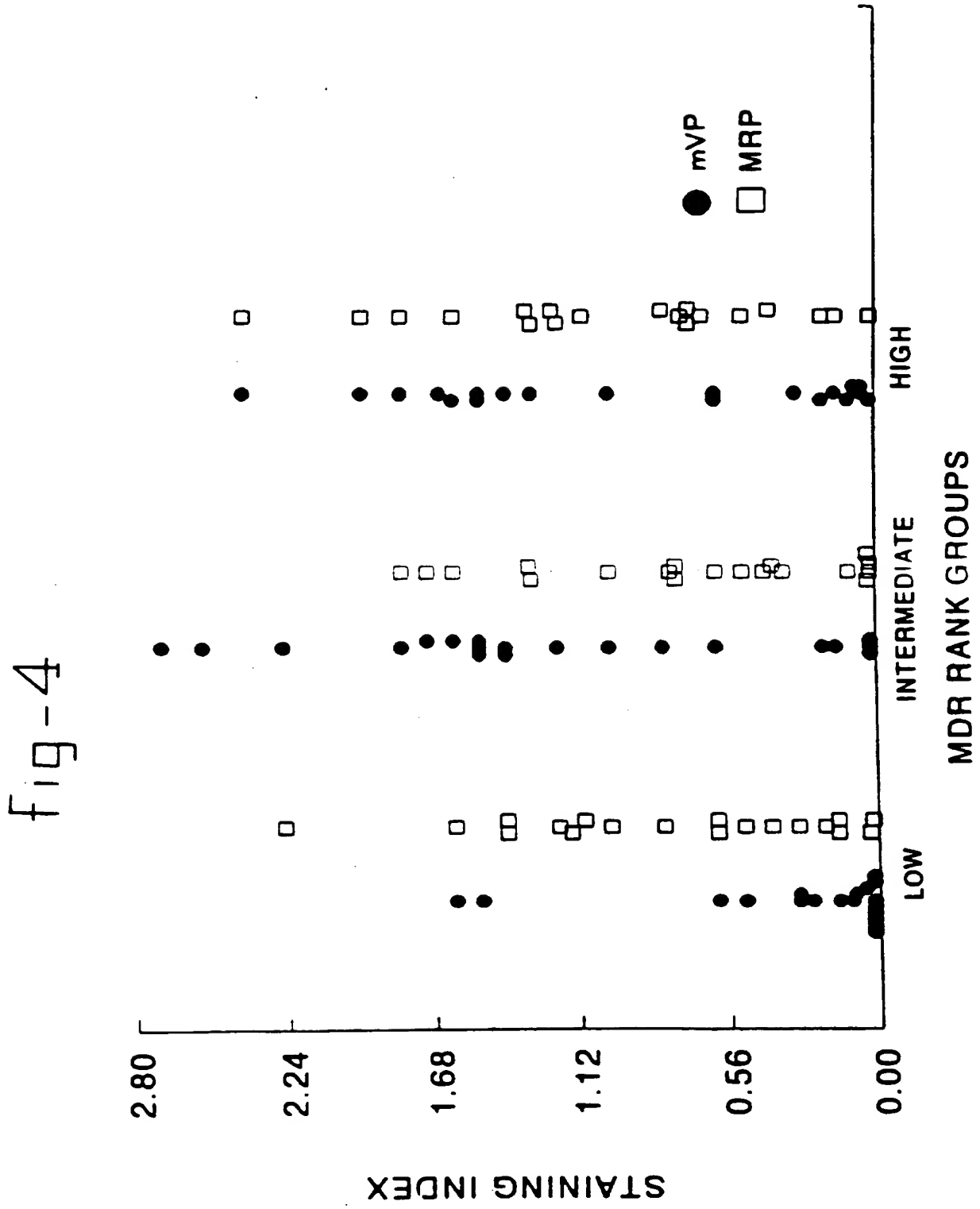


Fig-4

Figures 5/5

fig-5a

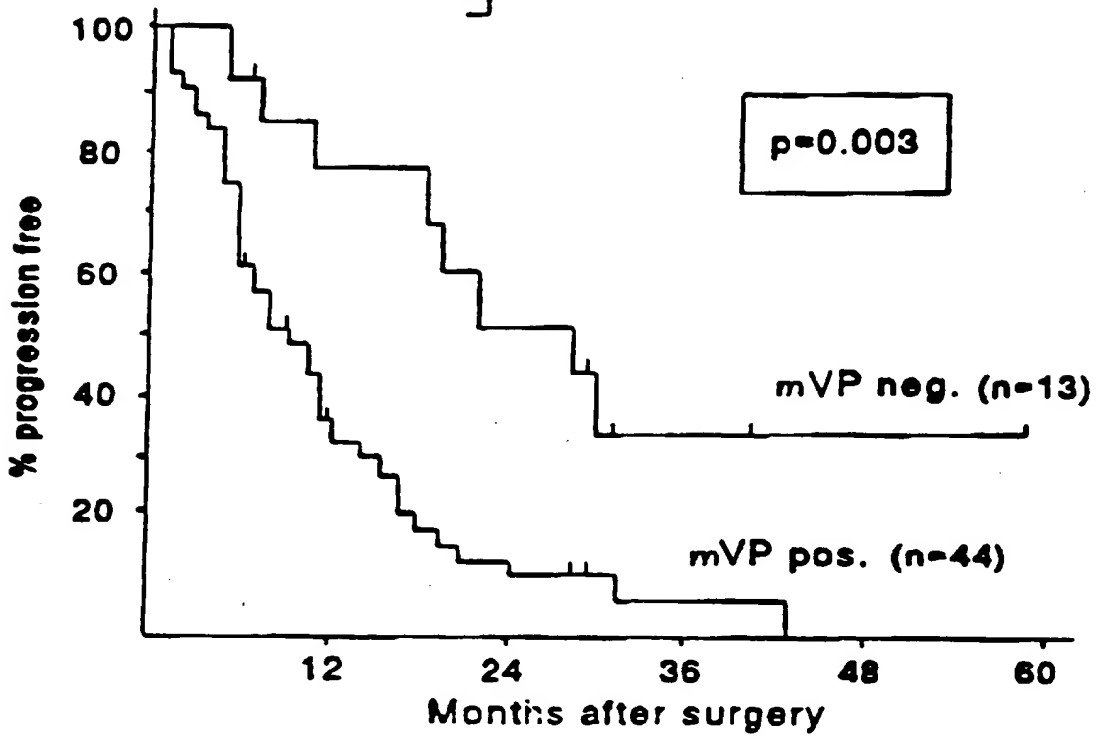
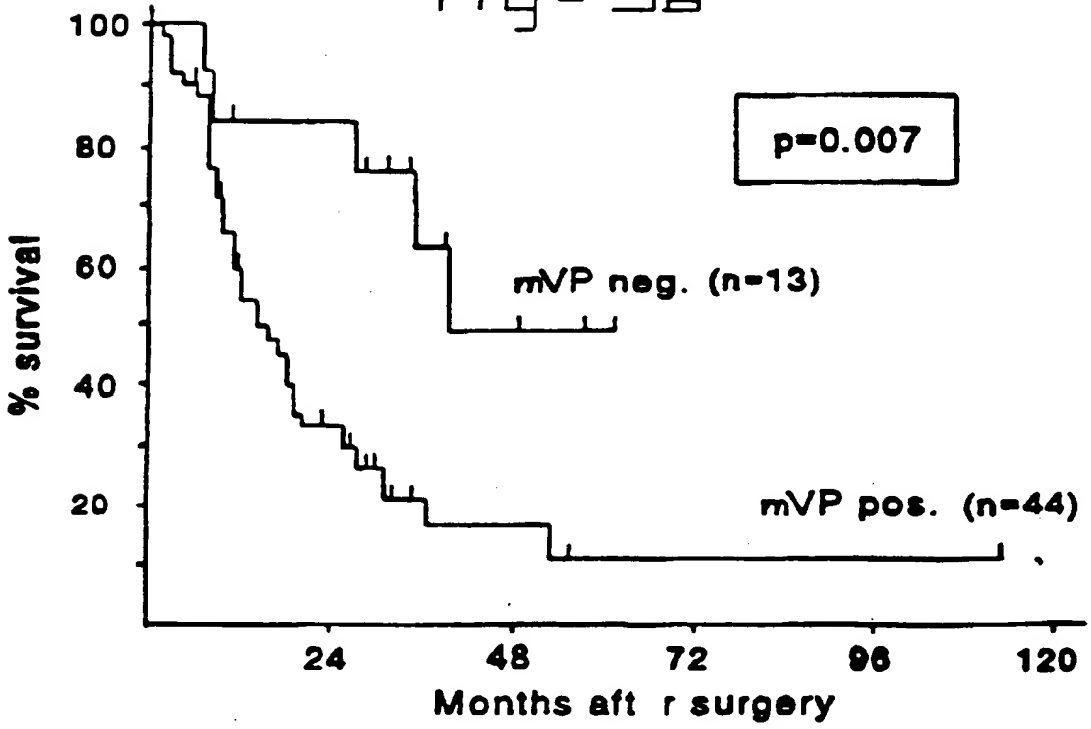


fig-5b



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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 96/01013

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/435 C07K16/18 G01N33/574 G01N33/569 C12Q1/68
C12P21/08 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH , vol. 35, March 1994, page 344 XP002002536 R.J. SCHEPER ET AL.: "Molecular and clinical characterization of the LRP protein associated with non-P-glycoprotein multidrug resistance." * Abstract nr. 2050 * --- -/--	1-5, 12-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 June 1996	11.07.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer De Kok, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RESEARCH, vol. 53, 1 April 1993, CHICOGO US, pages 1475-1479, XP002002537 R.J. SCHEPER ET AL.: "Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug-resistance" cited in the application see the whole document	1-4,16, 17
X	JOURNAL OF PATHOLOGY, vol. 175, no. 1, January 1995, CHICHESTER US, pages 13-22, XP002002538 P. RAMANI ET AL.: "Expression of mdr1/P-glycoprotein and p110 in neuroblastoma." see the whole document	1
X	GENOMICS, vol. 24, 1994, SAN DIEGO US, pages 276-279, XP002002648 K. SUDO ET AL.: "2058 expressed sequence tags (ESTs) from a human fetal lung cDNA library" see the whole document	12-15
A	GENE, vol. 151, 1994, AMSTERDAM NL, pages 257-260, XP002002539 V.A. KICKHOEFER ET AL.: "The sequence of a cDNA encoding the major vault protein from Rattus norvegicus" cited in the application see the whole document	12,13
A	TRENDS IN CELL BIOLOGY, vol. 1, no. 2/3, 1991, CAMBRIDGE GB, pages 47-50, XP002002540 L. ROME ET AL.: "Unlocking vaults: organelles in search of a function." cited in the application see the whole document	1
A	THE JOURNAL OF CELL BIOLOGY, vol. 110, no. 4, 1990, NEW YORK US, pages 895-901, XP002002541 N.L. KEDERSHA ET AL.: "Vaults. II. Ribonucleoprotein structures are higly conserved among higher and lower eukaryotes." cited in the application see the whole document	1-18

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BNSDOCID: <WO___9627611A1_I_>

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 9505 Derwent Publications Ltd., London, GB; AN 95-032328 XP002002546 & JP,A,06 315 375 (AGENCY OF IND SCI & TECHNOLOGY) , 15 November 1994 see abstract	8
A	EP,A,0 290 144 (CITY OF HOPE) 9 November 1988 see the whole document	1
P,X	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 36, March 1995, WASHINGTON US, page 323 XP002002542 G.L. SCHEFFER ET AL.: "The drug resistance related protein LRP is a major vault protein" * Abstract nr. 1921 *	1-18
P,X	NATURE MEDICINE, vol. 1, no. 6, June 1995, NEW YORK US, pages 578-582, XP002002543 G.L. SCHEFFER ET AL.: "The drug resistance related protein LRP is the human major vault protein." see the whole document	1-18
P,X	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 16, 16 August 1995, WASHINGTON US, pages 1230-1237, XP002002544 M.A. IZQUIERDO ET AL.: "Drug resistance-associated marker Lrp for prediction of response to chemotherapy and prognoses in advanced ovarian carcinoma" see the whole document	1-18
P,X	INTERNATIONAL JOURNAL OF CANCER, vol. 65, no. 2, 17 January 1996, NEW YORK US, pages 230-237, XP002002545 M.A. IZQUIERDO ET AL.: "Overlapping phenotypes of multidrug resistance among panels of human cancer-cell lines" see the whole document	1-18
P,X	EMBL Sequence Database, Accession number: N42270, 27 January 1996, L.Hillier et al., "cDNA clone human major vault protein" XP002002649 see the whole document	12-15

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BNSDOCID: <WO__9627611A1_I_>

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL Sequence Database, Accession number N32825, 13 January 1996, L.Hillier et al. "cDNA clone human major vault protein" XP002002650 see the whole document ---	12-15
P,X	EMBL Sequence Database, Accession number H65127, 20 October 1995, L. Hillier et al., "cDNA clone human major vault protein" XP002002733 see the whole document ---	12-15
L	EMBL Sequence Database, Accession number X79882, 6 July 1995, G.L.Scheffer et al., "H.sapiens LRP mRNA" XP002002651 see the whole document -----	12-15

1

BNSDOCID: <WO___9627611A1_I_>

ernational application No.

PCT/EP 96/01013

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6-11,19
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Reason: Lack of technical disclosure (Art.83 EPC)
Lack of support by description (Art. 84 EPC)

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/EP 96/01013

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0290144	09-11-88	AU-B-	1556188	10-11-88
		JP-A-	1055199	02-03-89
		US-A-	5166140	24-11-92

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